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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 39/10, 39/02, 39/00, A01N 63/00

A1

(11) International Publication Number: **WO 99/59630**

(43) International Publication Date: 25 November 1999 (25.11.99)

(21) International Application Number: PCT/US99/10690

(22) International Filing Date: 14 May 1999 (14.05.99)

(30) Priority Data:
60/085,691 15 May 1998 (15.05.98) US

(71) Applicant *(for all designated States except US)*: UNIVERSITY OF CALIFORNIA LOS ANGELES [US/US]; Office of Technology Transfer, 5th floor, 1111 Franklin Street, Oakland, CA 94607–5200 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MILLER, Jeff, F. [US/US]; 3469 Alana Drive, Sherman Oaks, CA 91403–4705 (US). HARVILL, Eric, T. [US/US]; 2491 Purdue Avenue #119, Los Angeles, CA 90064 (US). YUK, Ming, H. [SG/US]; 11811 Venice Boulevard #119, Los Angeles, CA 90066 (US). COTTER, Peggy, A. [US/US]; 20658 Martinez Street, Woodland Hills, CA 91364–2310 (US).

(74) Agents: WISE, Michael, J. et al.; Lyon & Lyon, Suite 4700, 633 West 5th Street, Los Angeles, CA 90071–2066 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: TYPE III *BORDETELLA* SECRETION SYSTEM

(57) Abstract

The invention relates to a *Bordetella* type III secretion system and its constituent components, genetically modified *Bordetella*, prophylactic and remedial live attenuated vaccines including genetically modified *Bordetella* and uses thereof.

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TYPE III *BORDETELLA* SECRETION SYSTEM

This application claims benefit under 35 U.S.C. § 119(e) to United States Provisional Application No. 60/085,691 filed May 15, 1998, incorporated herein by reference.

FIELD OF THE INVENTION

The invention relates to a *Bordetella* type III secretion system and its constituent components, genetically modified *Bordetella*, prophylactic and remedial live attenuated vaccines including genetically modified *Bordetella* and uses thereof.

BACKGROUND OF THE INVENTION

The genus *Bordetella* includes small, aerobic, Gram-negative coccobacilli associated with respiratory infections in humans and other animals. *B. pertussis* infects humans and causes whooping cough, a highly contagious disease with severe clinical manifestations in children (Hewlett, 1995). (Throughout this application various publications are referenced, the disclosures of these publications in their entireties are hereby incorporated by reference.) *B. parapertussis* causes a similar disease in humans and has also been recently isolated from sheep (Cullinane et al., 1987; Porter et al., 1994). *B. bronchiseptica* infects a broad range of mammals, and has been isolated from mice, rats, guinea pigs, rabbits, skunks, opossums, raccoons, cats, dogs, ferrets, foxes, pigs, hedgehogs, sheep, koala, bears, leopards and horses. Although bordetellosis is often asymptomatic, *B. bronchiseptica* can and does cause significant disease.

B. bronchiseptica and *Pasteurella multocida*, are a primary cause of infectious atrophic rhinitis in swine, a serious and widespread disease responsible for significant economic loss to the pork industry. Atrophic rhinitis is an upper respiratory disease that results in degeneration of the nasal turbinates, deviation of the nasal septum and atrophy of the nasal bone, which can be so severe that it causes visible deformation of the animal's snout. Pigs suffering from atrophic rhinitis feed less robustly and gain weight more slowly than healthy pigs, resulting in lower market weight and/or longer time-to-market. Atrophic rhinitis affects pigs worldwide causing significant global economic impact on the pork-producing industry.

Atrophic rhinitis is caused by co-infection of the swine upper respiratory tract with *B. bronchiseptica* and *Pasteurella multocida*. Results from both *in vivo* and *in vitro* studies indicate that colonization of the upper respiratory epithelium with *B. bronchiseptica*

predisposes pigs to infection by the opportunistic *P. multocida* which would otherwise be unable to efficiently establish infection. Although colonization by *B. bronchiseptica* alone may lead to pronounced turbinate atrophy, disease is most severe, sometimes progressing to pneumonia, when exacerbated by secondary infection with *P. multocida*. The inability of *P. multocida* to efficiently infect *Bordetella*-free swine suggests preventing infection with *Bordetella* would also prevent infection by *P. multocida* and the resulting atrophic rhinitis.

B. bronchiseptica is also responsible for causing infectious tracheobronchitis (ITB) (kennel cough) in dogs and a similar respiratory illness in cats. ITB has a worldwide distribution and commonly develops in dogs that are housed in groups. Because of its highly infectious nature, ITB is a major concern for breeding, boarding and training kennels as well as animal care facilities at research and training institutions. Although most facilities require dogs to be vaccinated against *B. bronchiseptica*, the efficacy of currently available vaccines is questionable at best and ITB remains a common and important canine disease. *B. bronchiseptica* also causes an upper respiratory disease in rabbits called snuffles. As with atrophic rhinitis, snuffles is exacerbated by co-infection with *P. multocida* and can progress to bronchopneumonia. Rats and mice are also natural hosts for *B. bronchiseptica* and are highly susceptible. The ID_{50} for infection with *B. bronchiseptica* in many cases is less than 20 cfu.

Bordetella spp. produce a number of protein factors (*e.g.*, virulence factors) that play important roles in the interaction between the bacteria and host cells, leading to establishment of infection, pathogenesis and transmission (Weiss and Hewlett, 1986). Protein factors involved in adhesion to host cells include filamentous hemagglutinin (FHA), pertactin and fimbriae (Mooi et al., 1992. Relman et al., 1989; Roberts et al., 1991); while production of toxins, including the bifunctional adenylate cyclase toxin/hemolysin (AC/HLY) (Hewlett and Gordon, 1988), dermonecrotic toxin (Walker and Weiss, 1994) and pertussis toxin (expressed only in B. pertussis) (Locht et al., 1986), are involved in the pathological effects on host tissues and the ability to evade host defenses.

Expression of these virulence factors is coordinately regulated by the bvgAS locus. BvgA and BvgS, which belong to the family of two-component signal transduction proteins found in prokaryotes and lower eukaryotes, are responsible for sensing external stimuli and coordinating the transcription of a large collection of genes and operons that function during the infectious cycle (Stibitz et al., 1988; Stibitz and Yang, 1991). Signal transduction is accomplished by a His-Asp-His-Asp phosphorelay mechanism (Uhl and Miller, 1994; Uhl and Miller, 1996; Uhl and Miller, 1996).

The Bvg^+ phase is characterized by expression of virulence factors and this phase is necessary and sufficient for colonization of rabbits and rats (Akerley et al., 1995; Cotter and Miller, 1994). The Bvg^- phase is avirulent and characterized by the loss of virulence gene expression and induction of genes which are repressed in the Bvg^+ phase, including motility genes in *B. bronchiseptica* (Akerley and Miller, 1993; Akerley et al., 1992).

There is a need for an effective *B. bronchiseptica* vaccine. Live attenuated vaccines, i.e., living viruses and bacteria that carry mutations rendering them avirulent or greatly reduced in virulence offer significant advantages in terms of manufacture and immunogenicity. A single inoculation of live vaccine at a modest dose may replicate *in vivo* to a large immunogenic dose and, during the course of replication, express the majority of immunogens seen during natural disease. The processing and presentation of these antigens more closely corresponds to that of infection. Live attenuated vaccines can induce mucosal immune responses, which are not efficiently elicited by systemically administered vaccines. The mode of delivery of the live attenuated vaccines is more simple, requiring ingestion or inhalation. Finally, because attenuated vaccines are living organisms, they may also be used as vectors by genetically engineering them to express heterologous antigens, thus providing a mechanism for protection from more than one disease.

Most infectious agents either infect mucosal surfaces directly or gain entry to the body via mucosae or mucosal lymphoid tissue. Immunization that elicits mucosal antibody is therefore a cost effective tool to prevent and combat infectious diseases. Mucosal immunity is believed to be dependent on the production of an IgA antibody response, which generally cannot be accomplished by conventional vaccine administration routes, for example injection of the antigen.

Live attenuated bacterial vaccines that generate effective mucosal immune responses have been successfully derived from modified bacteria of the genera *Mycobacterium*, *Salmonella*, *Shigella*, *Vibrio* and *Listeria*. The progress in the field over the last decade is reviewed in Killeen, et al., *Bacterial mucosal vaccines: Vibrio cholerae as a live attenuated vaccine/vector paradigm*, Curr Top Microbiol Immunol. 236:237-54 (1999). The goal of eliciting a mucosal immune response, in contrast to the elicitation of a systemic immune response in the absence of a mucosal immune response, is in principle necessary because the immune system is organized such that injected antigens are not effective in eliciting the generation of antibodies in what is known as the "common mucosal immune system."

The common mucosal immune system is an integrated system including gut-associated lymphoid tissue and nasal-associated lymphoid tissue. The common mucosal

immune system is stimulated by antigens entering central inductive sites present in mucosal tissues, where they interact with accessory and lymphoid cells and stimulate specific IgA-committed B cells and appropriate T cells. These cells then migrate to effector sites, including the lamina propria of the gastrointestinal and respiratory tracts as well as to remote effector sites, such as the exocrine glands, including the salivary, mammary and lacrimal glands. At the effector sites, surface IgA+ B cells clonally expand and terminally differentiate into IgA-secreting plasma cells under the influence of antigen, T helper cells and cytokines, such as interleukin (IL)-5, IL-6, and IL-10.

Live attenuated strains of pathogenic bacteria have proven uniquely successful in generating protective immune responses against the wild type pathogenic bacteria at mucosal sites. The live attenuated strains have also proven very effective in generating protective immune responses against other pathogens, particularly when the live attenuated bacteria are genetically engineered to express antigenic epitopes of these other pathogens.

Two modes of attenuation have been used: undefined mutations and defined mutations. The most widely used attenuated bacterial vaccines - *Salmonella typhi* strain Ty21a and *Mycobacterium bovis* strain Bacille, Calmette, Guerin (BCG) - were attenuated by undefined mutations. BCG is widely used throughout the world as a live vaccine for tuberculosis and in the treatment of certain cancers. *S. typhi* Ty21a is administered in three or four oral doses and is very well tolerated in humans. The immune response elicited is both humoral and cell-mediated and confers significant, but incomplete, protection from typhoid fever.

Numerous attenuated strains of *Vibrio*, *Salmonella*, and *Shigella* that carry defined attenuating mutations have also been generated. These defined genetic mutations have been of two subclasses (1) disruption of gene(s) affecting metabolism or regulation and (2) disruption of gene(s) affecting virulence. While the metabolically attenuated strains proved unsuccessful either due to their inability to provoke significant immune responses or because they caused unacceptable levels of high fever and bacteremia, virulence attenuation has proven to be a highly successful strategy. Killeen, et al., at 240-243. Safer and more effective vaccines have thus been constructed by virulence attenuation.

Moreover, the successful genetic attenuation of live bacteria resulting in safe, immunogenic and protective vaccines has enabled the development of safe vaccine vectors. The use of genetically attenuated live bacteria as vaccine vectors has proven successful against viral, bacterial and parasitic derived pathogens. BCG, for example, has vectored several bacterial and viral antigens from Borrelia, pneumococci, simian immunodeficiency

virus (SIV) and human immunodeficiency virus (HIV), eliciting immune responses against each antigen when used to immunize mice. BCG vectored outer-surface protein A (OspA) and pneumococcal-surface protein A (PspA) conferred protection against *Borrelia* and *S. pneumoniae*, respectively in murine challenge models.

Attenuated *Salmonella* have proven capable of delivering more than 30 bacterial, 10 viral and 10 parasitic antigens in pre-clinical murine studies. Kaufmann, Concepts in vaccine development, de Gruyter, Berlin (1996). Protective immunity was demonstrated with bacterial challenge with *Y. pestis*, *L. monocytogenes* and *B. pertussis*, viral challenge with Herpes simplex virus and influenza, parasitic challenge by *L. major* and *S. mansoni*, among others. Kaufmann, Concepts in vaccine development, de Gruyter, Berlin (1996).

Listeria monocytogenes has also been successfully used as a live attenuated vaccine vector. *See, e.g.*, Shen H, et al., *Compartmentalization of bacterial antigens: differential effects on priming of CD8 T cells and protective immunity*, Cell. 92(4):535-45 (1998); Jensen ER, et al., *Recombinant Listeria monocytogenes vaccination eliminates papillomavirus-induced tumors and prevents papilloma formation from viral DNA*, J Virol. 71(11):8467-74 (1997); Jensen ER, et al., *Recombinant Listeria monocytogenes as a live vaccine vehicle and a probe for studying cell-mediated immunity*, Immunol Rev. 158:147-57 (1997); Slifka MK, et al., *Antiviral cytotoxic T-cell memory by vaccination with recombinant Listeria monocytogenes*, J Virol. 70(5):2902-10 (1996); Shen H, et al., *Recombinant Listeria monocytogenes as a live vaccine vehicle for the induction of protective anti-viral cell-mediated immunity*, Proc Natl Acad Sci U S A. 92(9):3987-91 (1995).

SUMMARY OF THE INVENTION

In a first, independent aspect of the present invention, a *Bordetella* type III secretion system and its constituent components is disclosed.

In a second, independent aspect of the present invention, genetically engineered *Bordetella* having modifications of the type III secretion system and its constituent components is disclosed.

In a third, independent aspect of the present invention, live genetically attenuated *Bordetella bronchiseptica* having modifications of the type III secretion system or its constituent components effective in preventing infection with wild type *Bordetella bronchiseptica* is disclosed.

In a fourth, independent aspect of the present invention, a vaccine including genetically engineered *Bordetella bronchiseptica* having modifications of the type III

secretion system and/or its constituent components effective in preventing infection with wild type *Bordetella bronchiseptica* is disclosed.

In a fifth, independent aspect of the present invention, methods are disclosed for using genetically engineered *Bordetella bronchiseptica* having modifications of the type III secretion system as a vaccine against wild type *Bordetella bronchiseptica.*

In a sixth, independent aspect of the present invention, genetically engineered, attenuated *Bordetella* expressing a heterologous antigen effective as a live mucosal antigen delivery vector is disclosed.

In a seventh, independent aspect of the present invention, genetically engineered *Bordetella* expressing a heterologous antigen effective in generating a mucosal immune response to the heterologous antigen is disclosed.

In an eight, independent aspect of the present invention, a vaccine including *Bordetella* expressing a heterologous protective antigen effective in generating a mucosal immune response to the heterologous antigen is disclosed.

In a ninth, independent aspect of the present invention, methods are disclosed for using *Bordetella* expressing a heterologous antigen to generate a mucosal immune response to the heterologous antigen.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Differential display PCR with primers KB4 and ML2 (see Table 2) identified fragments produced only from the Bvg^+ but not Bvg^- phase cDNA. cDNAs made from total RNA of *B. bronchiseptica* strain RB50 grown in the Bvg^+ phase (lanes 1, 2, 5, 6) or Bvg^- phase (lanes 3, 4, 7, 8) were used as templates for PCR with primer KB4 (lanes 1 to 4) or primer ML2 (lanes 5 to 8). Each reaction was done in duplicate except that the odd numbered lanes contain twice the amount of cDNA template as the even numbered lanes. Arrows indicate positions of prominent bands amplified only from Bvg^+ phase cDNA.

Figure 2: Protein sequence of BscN compared to YscN. The two conserved Walker Boxes are outlined, and the shaded region is deleted in the mutant strain WD3.

Figure 3. Genomic organization and open reading frames (arrows) of type III secretion genes in *B. bronchiseptica*. The numbers below each open reading frame indicate the percentage amino acid identity to corresponding homologues in (1) *Yersinia spp.* and (2) *Pseudomonas aeruginosa*. The letters A, B and C denote ORFs of secreted proteins identified in figure 5.

Figure 4: *BscN* expression is regulated by *bvg* and it is expressed in the Bvg^+ phase. a. RT-PCR with primers specific for *fhaB*, *fla*A and *bscN*. Genomic DNA (g) and cDNAs made from total RNA of *B. bronchiseptica* strain RB50 grown in Bvg^+ phase (+) or Bvg^- phase (-) were used as templates for PCR. Controls of mock RT were templates in which the RT reactions were done in the absence of reverse-transcriptase. Primer pairs specific for *fhaB* (NX7+KX31), *flaA* (BA04+BA011) and two separate regions of *bscN* (W1+W2 and W3+W4) were used for PCR. See Table 2 for sequences of primers. b. RT-PCR with the same primers as in part a, but with genomic (g), cDNAs and mock RT templates from phase-locked strains of *B. bronchiseptica* RB53 (locked in Bvg^+ phase) and RB54 (locked in Bvg^- phase). c. Slot blots of total RNA with probes for *fhaB*, *flaA*, *recA* and *bscN*. ^{32}P labeled DNA probes were used to detect specific transcripts in total RNA isolated from RB50 grown in Bvg^+ phase (+) or Bvg^- phase (-). RNA samples were diluted two-fold and eight-fold in the second and third rows respectively of each panel.

Figure 5: In-frame deletion of *bscN* causes decreased secretion of certain polypeptides from *B. bronchiseptica in vitro*. TCA precipitates of proteins from culture supernatants of wild type strain RB50 (lanes 1, 3, 6, 8, 10, 12), *bscN* deletion strain WD3 (lanes 2, 4, 7, 9, 11, 13) and Bvg^- phased-locked strain RB54 (lane 5) were separated by 4 to 12% gradient SDS-PAGE and detected by: Coomassie stain (lanes 1 and 2); immunoblots with antisera from rabbit (lanes 3 to 5), rat (lanes 6, 7, 10, 11) and mouse (lanes 8, 9, 12, 13), which were previously infected with RB50 (lanes 3 to 9) or WD3 (lanes 10 to 13). Open arrows indicate major proteins produced from RB50 but not WD3 as detected by Coomassie staining while solid arrows indicate major proteins produced only from RB50 as detected by immunoblots. Numbers on the right column indicate positions of molecular weight markers in kiloDaltons. Figure 5' is another reproduction of the photograph in figure 5. Polypeptides indicated as A, B and C were electroblotted onto PVDF paper and their amino-terminal sequences determined by Edman degradation. Amino terminal sequence of A was determined as: XRIDAARNPXHAAMQ. Amino terminal sequence of B was determined as: SVSPTSPGSFGAGPV. Amino terminal sequence of C was determined as: TIDLGVSLTSQAGGL. Three ORFs that correspond to these amino-terminal sequences are indicated in figure 3.

Figure 6: Deletion of *bscN* leads to decreased cytotoxicity of *B. bronchiseptica* on L2 epithelial cells. L2 cells were incubated with *B. bronchiseptica* strains RB50 (b and c), WD3

(e and f) and FF1 (d) with MOI of 500 or without any bacteria (a) and incubated for 15 minutes (b and e) or two hours (c, d and f). Cells were washed, fixed, stained with Giemsa stain, and observed under microscope.

Figure 7: Deletion of *bscN* eliminates tyrosine dephosphorylation of certain proteins in L2 cells upon infection with *B. bronchiseptica*. Confluent L2 cells were incubated with *B. bronchiseptica* strains RB50 (lanes 3 and 4), WD3 (lane 5) and FF1 (lane 6) with MOI of 500 or without any bacteria (lane 2) for 1 hour. 1 mM vanadate was added to one sample of L2 cells incubated with RB50 (lane 4). Total cell protein from RB50 itself (lane 1) and each of the above samples of infected L2 cells were separated by 8% SDS-PAGE and probed with monoclonal antibody PT-66 specific for phosphotyrosine. Arrows indicate tyrosine-phosphorylated proteins that are apparently dephosphorylated upon incubation with RB50, but not with WD3 or FF1.

Figure 8: Deletion of *bscN* causes defect in persistence of *B. bronchiseptica* in the trachea of rats. Female 4 week old Wistar rats were inoculated intranasally with 1000 cfu of either RB50 or WD3 in a 5µl suspension. Groups of 4 animals were sacrificed 14 and 35 days post-inoculation. The nasal septum and 1 cm of the trachea were homogenized and plated on BG agar to determine recoverable cfu of *B. bronchiseptica* from these tissues of each animal. Differences between cfus of RB50 and WD3 recovered from the nasal septum on days 14 and 35, or from the trachea at day 14, were not statistically significant. Dotted line indicates the minimum level of detection.

Figure 9: *BscN* is not transcribed in most *B. pertussis* strains and a human isolate of *B. parapertussis*. Genomic DNA (g) and cDNAs from total RNA of Bvg^+ phase (+) and Bvg^- phase (-) cells of the following strains were used as templates for PCR: *B. bronchiseptica* RB50; *B. pertussis* strains GMT1, 17471, Tohama 1, 18323; human isolate of *B. parapertussis* strain A168 and ovine isolate of *B. parapertussis* strain Hl. Primers specific for *bscN* in RB50 (W3 + W4) were used for PCR of the genomic DNA, cDNAs and also mock RT samples (0).

Figure 10. In-frame deletion of *bsp22* does not affect secretion of other type III secreted polypeptides. TCA precipitates of 1 ml of culture supernatant of wild type (RB50), *ΔbscN*

(WD3) and *Δbsp22* (D218) strains of *B. bronchiseptica* were immunoblotted with mouse antiserum specific for Bsp22 (A) antisera from a rabbit previously infected with RB50 (B). Thick arrow indicates Bsp22, thin arrows indicate other as yet unidentified type III secreted polypeptides, which are not secreted by WD3. Bsp22 secretion is restored in D218 by the presence of plasmid pLB2, which contains the ORF of *bsp22* in the broad host range vector pBBR1MCS (A, right lane).

Figure 11. Disruption of type III secretion leads to more rapid clearance of *B. bronchiseptica* from the trachea of C57BL/6 mice (A) and reduced colonization of the trachea of BALB/c mice (B). Female 4 week old mice were inoculated intranasally with 10^5 cfu of wild type (RB50), *ΔbscN* (WD3) or *Δbsp22* (D218) strains in a 50 μl suspension. Groups of 3 to 4 animals were sacrificed 7 and 35 days post- inoculation. The nasal extract and 0.5 cm of the trachea of each animal were homogenized and plated on BG agar to determine recoverable cfu of *B. bronchiseptica* from these tissues of each animal. Dotted line indicates the minimum level of detection. Horizontal bars indicate means of cfu counts. Recovered cfu from the trachea showed significant difference (*) between that of RB50 and WD3 infected BALB/c mice on day 35 ($p=0.03$). Recovered cfu from the trachea also showed significant difference (*) between that of RB50 and D218 infected BALB/c mice on day 35 ($p=0.01$).

Figure 12. Type III secretion mutants elicit higher titers of anti-*Bordetella* antibodies in infected mice compared to wild type infected animals. Wild type bacterial cells were coated onto ELISA plates and probed with serial dilutions of sera from BALB/c (A) or C57BL/6 (B) mice infected with RB50 or WD3 or D218 for 35 days. Bound anti-*Bordetella* antibodies were detected by secondary antibodies specific for total immunoglobulins. Results were averages from 3 or 4 animals in each group. * indicates statistically significant difference in titers between serum from wild type infected host and that from type III secretion mutant infected host at $p<0.05$.

Figure 13. Disruption of type III secretion causes increase in virulence of *B. bronchiseptica* in SCID-beige mice. (A) 500 cfu of RB50, WD3, D218 or RB54 (*Δbvg*) in 5 μl droplets were inoculated into the nasal cavity of groups of 10 SCID-beige mice. Health of the mice was monitored over a three month period and morbid mice were sacrificed prior to death. Time of survival of the mice inoculated with RB50 (circles) were significantly (as determined by

Logrank analysis) longer than those infected with WD3 (triangles) (Chisquare= 9.701, P-value=0.0018) or D218 (squares) (Chi-square= 9.605, P-value=0.0019). (B) Growth curve of RB50, WD3 and D218 in Stainer-Scholte medium at 37°C with aeration as determined by optical density of liquid culture. (C) Similar numbers of bacteria could be recovered from SCID-beige mice infected with wild type or type III secretion defective strains of *B. bronchiseptica* in early stages of infection. $5x10^5$ cfu of RB50, WD3 or D218 were inoculated into groups of 3 to 4 SCID-beige mice and animals were sacrificed after 5 days. Recoverable cfu from the nasal cavities and lungs of animals were determined and compared. Unpaired t-tests showed no significant differences in the recovered cfu from each organ among different bacterial strains.

Figure 14. Cytotoxicity of *B. bronchiseptica* towards macrophage-like cell lines *in vitro* depends mostly on type III secretion. RB50 (wild type), WD3 (*ΔbscN*), D218 (*Δbsp22*) or RB54 (Bvg^- phase-locked mutant) were incubated with J774A.1 macrophage-like cell lines at MOI of 10 for 2 hours. Cytotoxicity is determined by release of lactate dehydrogenase as measured with the CytoTox96 kit and represented as arbitrary units.

Figure 15. Type III secretion is required for induction of apoptosis of J774A.1 mouse macrophage cell lines by *B. bronchiseptica*. J774A.1 cells were infected with wild type strain RB50 (A), *ΔbscN* strain WD3 (B), or *Δbsp22* strain D218 (C) with MOI of 50 for 60 minutes, then washed, fixed, permeabilized and incubated with fluorescent TUNEL reagent according to manufacturer's protocol. Each sample was observed by differential interference contrast microscopy (top row) or epifluorescent microscopy (bottom row).

Figure 16. Type III secretion is required for induction of apoptosis by *B. bronchiseptica in vivo*. Female 4 week old BALB/c mice were inoculated intranasally with 10^5 cfti of PBS (A), RB50 (B) or WD3 (C) in a 50 µl suspension. The animals were sacrificed after 2 days and their lungs were fixed and sectioned as described in experimental procedures. The sections were then deparaffinized, labeled with fluorescent TUNEL reagent according to manufacturer's protocol and observed under phase-contrast epifluorescent microscope. Images are overlays of phase-contrast and fluorescent images of the same view.

Figure 17. Type III secretion by *B. bronchiseptica* is required for aggregation and inactivation of *NF-κB* in L2 cells *in vitro*. The rat lung epithelial cell line L2 was incubated with: (A) medium alone; (B) medium with 10 ng/mlTNFα for 10 minutes; (C) wild type *B. bronchiseptica* (RB50) at MOI of 100 for 20 minutes; (D) wild type *B. bronchiseptica* (RB50) at MOI of 100 for 20 minutes, followed by 10 ng/ml TNFα for 10 minutes; (E) *ΔbscN* strain of *B. bronchiseptica* (WD3) at MOI of 100 for 20 minutes; (F) *ΔbscN* strain of *B. bronchiseptica* (WD3) at MOI of 100 for 20 minutes, followed by 10 ng/ml TNFα for 10 minutes. Cells were fixed, permeabilized, labeled with antibody specific for *NF-κB* p65 subunit and observed by epifluorescent microscopy as described in materials and methods.

Figure 18. DNA sequence corresponding to figure 3. Seq. ID 1.

Figure 19. DNA sequence corresponding to ORF of bscV. Seq. ID 2.

Figure 20. DNA sequence corresponding to ORF of bcr3/bscX. Seq. ID 3.

Figure 21. DNA sequence corresponding to ORF of bopN. Seq. ID 4.

Figure 22. DNA sequence corresponding to ORF of bsp22. Seq. ID 5.

Figure 23. DNA sequence corresponding to ORF of bcrH1. Seq. ID 6.

Figure 24. DNA sequence corresponding to ORF of bopD. Seq. ID 7.

Figure 25. DNA sequence corresponding to ORF of bopB. Seq. ID 8.

Figure 26. DNA sequence corresponding to ORF of bcrH2. Seq. ID 9.

Figure 27. DNA sequence corresponding to ORF of bcr4/bscY. Seq. ID 10.

Figure 28. DNA sequence corresponding to ORF of bscI. Seq. ID 11.

Figure 29. DNA sequence corresponding to ORF of bscJ. Seq. ID 12.

Figure 30. DNA sequence corresponding to ORF of bscK. Seq. ID 13.

Figure 31. DNA sequence corresponding to ORF of bscL. Seq. ID 14.

Figure 32. DNA sequence corresponding to ORF of bscN. Seq. ID 15.

Figure 33. DNA sequence corresponding to ORF of bscO. Seq. ID 16.

DETAILED DESCRIPTION OF THE INVENTION

In one embodiment, the invention provides a type III secretion system in *Bordetella*. This system comprises multiple proteins encoded by nucleic acid sequences that comprise the type III secretion system for *Bordetella*. In one embodiment, the DNA sequence of Fig. 18 is provided. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bscV corresponding to Figure 19. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bcr3/bscX corresponding to Figure 20. In another embodiment, one

protein of the type III secretion system is encoded by a nucleic acid sequence designated bopN corresponding to Figure 21. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bsp22 corresponding to Figure 22. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bcrH1 corresponding to Figure 23. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bopD corresponding to Figure 24. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bopB corresponding to Figure 25. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bcrH2. corresponding to Figure 26. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bcr4/bscY corresponding to Figure 27. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bscI corresponding to Figure 28. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bscJ corresponding to Figure 29. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bscK corresponding to Figure 30. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bscL corresponding to Figure 31. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bscN corresponding to Figure 32. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bscO corresponding to Figure 33.

Further, nucleic acid sequences are provided that hybridize (e.g., under stringent conditions) to the nucleic acid sequences of the invention, including, for example, the homologous genes in *B. pertussis* and *B. parapertussis*. Additionally, the invention provides polypeptides encoded by the nucleic acid molecules of the invention.

Bordetella spp. attenuated in accordance with the present invention are effective bacterial vectors for immunization against *Bordetella* infection as well as against heterologous antigens.

In accordance with the present invention, Type III secretion system includes the "core components" that form the apparatus in the wild type bacteria that injects factors into host cells or the surrounding medium, "effectors," which are transported by the core apparatus into the host cells or into the surrounding medium and "accessory factors" including chaperones

and other components used by the wild type bacteria to control and effectuate the production and delivery of the effectors by the core apparatus.

Type III secretion system modifications in accordance with the present invention include any mutation that diminishes, abolishes or otherwise alters the effectiveness of the type III secretion system in performing any of the functions it carries *in vivo*. These modifications include, for example, mutations to the "core" proteins that decrease or abolish the ability of the system to secrete proteins or to translocate effectors into host cells or host cell membranes, mutations deleting or modifying effector genes, such that they are not produced or their function is attenuated, or mutations to other components of the system, chaperones for example, which are necessary for the delivery of the effectors in the wild type bacteria. Regulatory elements, transcription factors and other components used by the wild type bacteria may also be altered in such manner that the transcription, translation and/or processing of a component or components of the system is altered. *Bordetella* genetically engineered to include these mutations are also considered novel.

The generation of mutants is well known in the art. Loci coding for type III secretion system proteins or polypeptides may be altered in any effective manner. Preferred are deletions of the coding region for a particular protein or polypeptide, or the coding region of several proteins or polypeptides. Alternatively, an entire operon coding for such proteins and/or polypeptides may be deleted. Also preferred are in frame deletion or deletions of one or more codons of a protein or polypeptide, such that the function of the protein or polypeptide is abolished or attenuated. Also preferred is the substitution of one or more codons in one (or more) protein(s) or polypeptide(s) such that the function of the protein(s) or polypeptide(s) is abolished or altered. Also preferred are insertional mutations. Combinations thereof may also be used.

Another aspect of the present invention is the use of genetically modified *Bordetella*, preferably *B. bronchiseptica*, as an effective mucosal antigen-delivery system, and as the component of a vaccine. Delivery of antigens to mucosal surfaces by expressing the antigens in the bacteria of the present invention is effective for inducing mucosal immunity. Systemic immunity may also be provided. The resulting immunity serves to protect the host from microbial, and the like, invasion, or to combat such organisms when present in the host.

When used as an antigen delivery system, *Bordetella* are genetically modified such that they may express one or more heterologous antigens. Any *Bordetella* may be used as a live vector, however *Bordetella* attenuated in accordance with the present invention are preferred. Most preferred are recombinant attenuated *Bordetella bronchiseptica*. In

accordance with the present invention, *Bordetella* for use as a vaccine component or antigen delivery system may be attenuated by modifications to components other than to the type III secretion system, which nonetheless decrease or abolish export of type III secretion factors. For example, the strain Frl^r described in Akerley, et al., *Ectopic expression of the flagellar regulon alters development of the Bordetella-host interaction*, Cell 80(4):611-20 (1995), was engineered such that the *frlAB* promoter was replaced with that of *fhaB*. The entire motility regulon and flagellar proteins were expressed in the Bvg$^+$ rather than the Bvg$^-$ phase. This mutant has now been discovered to not secrete most, if not all, type III wild type secreted proteins. The use of these modified *Bordetella*, and the like, as antigen delivery systems and as vaccine components is also considered novel.

The bacteria may also be further modified such that they do not express other toxic factors, for example hemolysin/adenylate cyclase toxin, dermonecrotic toxin, and the like. They may also be modified such that their transmissibility is attenuated or abolished, for example by mutating genes necessary for the Bvg$^-$ phase, including motility genes, the *frlAb* locus, which encodes two proteins that function together as a transcriptional activator that regulates the motility regulon, the urase gene, the alcaligin gene, and the like. Mutants that are Bvg$^+$ phase-locked may also be used. As a precaution, DNA repair and DNA recombination enzymes, mating factors and accessory proteins, sites for integration of heterologous DNA, for plasmids or phages, for example, may also be altered such that the risk of the modified *Bordetella* exchanging or incorporating DNA from other organisms in the environment are decreased.

Bordetella may be modified in any suitable manner such that it expresses the heterologous antigen. The DNA encoding the heterologous antigen may be present extra-chromosomally, but is preferably integrated into the bacterial chromosome. The construct may include regulatory sequences, ribosome binding sequences, and other elements necessary for the proper expression of the antigen or antigens. The construction and manipulation of these expression vectors are well known to those of skill in the art.

When the DNA encoding the heterologous antigen is present extra-chromosomally, for example in a plasmid, a balanced lethal host-vector system strategy may be used. *See, e.g.*, Curtiss, et al., *Stabilization of Recombinant Avirulent Vaccine Strains in Vivo*, Res Microbiol 141:797-805 (1993) (incorporated herein by reference). This strategy is preferred when the preservation of the plasmid and the expression of the antigen for prolonged periods is desired.

The antigens may be expressed under the control of any effective promoter, and may include other regulatory sequences and sequences necessary for the appropriate translation of the gene product. For example, constitutive promoters may be used. The use of the promoter for the recA gene (Kuhl, et al., *Isolation and characterization of the recA gene of Bordetella pertussis*, Mol Microbiol 7:1165-72 (1990)) is preferred. Alternatively, antigens may be expressed under the control of inducible promoters, which are also well known in the art. Preferred is the use of the inducible Tac promoter (Walker, et al., *Construction of minitransposons for constitutive and inducible expression of pertussis toxin in bvg-negative Bordetella Bronchiseptica*, Inf & Immun 59:4238-48). Preferred is the expression of the antigen(s) under the control of the *bvgAS* locus, which controls the expression of virulence factors in wild type *Bordetella*. The use of the regulatory sequences used by wild type *Bordetella* for the expression of virulence factors is most preferred. The construct may also be inserted in a functional manner into an operon having any of the above described characteristics.

Any antigen may be expressed, including multiple antigens. The antigen(s) may be expressed such that it remains within the bacteria, or may have a leader sequence such that the antigen is secreted, a transmembrane sequence or sequences, such that the antigen is anchored to the bacterial cytoplasmic or outer membranes, or a sequence such that it is injected via the type III secretion system into the host cells. Combinations may also be used, such that the same antigen is displayed in the membrane, secreted, and/or expressed within the bacteria. When multiple antigens are expressed, they may be processed differently.

The antigens may also be expressed as fusion proteins. *See, e.g.*, Staats, et al., *Mucosal immunity to infection with implications for vaccine development*, Curr. Opin. Immunol 6:572-583 (1994) and references cited therein. Any effective fusion protein may be used. For example, for secretion the antigen may be fused to a protein or a portion of a protein that is secreted. Preferred are fusions to filamentous hemagglutinin, hemolysin/adenylate cyclase toxin, and the like, and portions thereof. For integration into the membrane, the antigen may be fused to any membrane protein. Preferred is the use of pertactin. For secretion via the type III secretion system the antigen may be fused to a protein secreted via the type III secretion system, and in particular it is desirable that about the first 10-20 codons of the secreted protein be present. *See, e.g.*, Anderson, et al., Yersinia enterocolitica *type III secretion: an mRNA signal that couples translation and secretion of YopQ*, Mol Microbiol 31(4):1139-48 (1999). The antigen may also be fused to other antigens, or to proteins known to have an adjuvant, or other desirable effect.

A protein or polypeptide, including for example an antigen, may be expressed as a fusion to a type III secreted gene so that it may be targeted into the host cytoplasm or membrane, or the extracellular medium by translocation of the fusion product via the type III secretion system. The strain expressing this construct may also be attenuated by specific inactivation of other type III secreted products (but not the "core" secretion apparatus genes) so that the only protein targeted into the host cell by type III secretion system is that of the fusion construct.

Antigens derived from *Leptospira canicola*, *L. grippotyphosa*, *L. hardjo*, *L. icterohaemorrhagiae*, *L. pomona*, *L. interrogans*, *L. bratislava*, canine distemper virus, canine adenovirus type 2, canine parainfluenza virus, canine parvovirus, rabies, herpes viruses, HIV, SIV, *Erysipelothrix rhusiopathiae*, *Pasteurella*, *P. multocida*, *Ascaris*, *Oesophagostomum*, pseudorabies virus, porcine parvovirus, pathogenic *E. coli*, including *E. coli* having K88, K99, 987P, and/or F41 adherence factors, *Clostridium spp.*, including *Cl. perfringens*, and *Cl. perfringens* type C beta toxoid, *Salmonella*, *Vibrio*, *Mycoplasma*, *Actinobacillus pleuropneumoniae*, *Haemophilus*, rotavirus, transmissible gastroenteritis virus, *Streptococcus sobrinus*, *S. mutans*, influenza, to name a few, may be used. Other antigens, and antigens from other pathogens, which may be used in accordance with the present invention are within the skill and knowledge in the art. *Bordetella* factors, including virulence factors, may also be expressed, preferably in a modified form which prevents their deleterious effects while permitting the elicitation of an immune response specific to those factors.

Antigens expressed may be in the form of whole heterologous proteins, or portions thereof, and may be modified such that their toxicity is decreased, their stability increased, or such that the protein or polypeptide may have other suitable or desirable modifications. The expression of polypeptides consisting of protective epitopes is preferred. The antigen may be derived from any pathogen, toxin, or other immunogenic material, including viruses, bacteria, eukaryotes, unicellular and multicellular parasites, and combinations thereof, or small molecules may be produced by the expression of the appropriate enzymes in the modified *Bordetella*. As most agents either infect mucosal surfaces directly or gain entry to the body via mucosae or mucosal lymphoid tissue, immunization that elicits mucosal antibodies is effective in preventing and combating these agents.

The bacteria may also be engineered to directly or indirectly produce inflammatory stimuli. Direct inflammatory stimuli may be produced, for example, by the expression and secretion of pro-inflammatory cytokines (for a review, see Svanborg, et al., *Cytokine*

responses during mucosal infections: role in disease pathogenesis and host defence, Curr Opin Microbiol 2(1):99-105 (1999)), including TNF, IL-1, IL-2, and other interleukins, TGF-β, interferon (for example INF-γ), GM-CSF, and the like, NO inhibitors (*see, e.g.*, Thepen et al., *The role of alveolar macrophages in regulation of lung inflammation*, Ann N Y Acad Sci. 725:200-6 (1994)), and the like. Enzymes or other proteins or polypeptides that may advantageously augment the immune response may be also be expressed.

In general, the genetically modified *Bordetella* may be used as an antigen delivery system in any animal that will support the modified *Bordetella* for a time sufficient to elicit an immune response. These include, for example, humans, dogs, cats, pigs, cows, sheep mice, rats, guinea pigs, rabbits, skunks, opossums, raccoons, ferrets, foxes, hedgehogs, koala, bears, leopards, horses and other animals.

The live attenuated *Bordetella* and the bacterial vectors of the present invention may be administered by any effective route. They are preferably administered such that the bacteria colonize the nasal mucosa. The trachea may also be colonized, albeit at times only temporarily. Preferred is direct administration to the nasal mucosa, by spraying or injecting the bacteria suspended in a suitable solution, for example. Suitable solutions include water, saline, and other solutions known in the art. Inhalation and oral administration are also preferred.

In accordance with the present invention, the live attenuated *Bordetella* may be administered in any effective manner. The nasal route of administration is preferred. The reduced quantity of proteolytic enzymes and reduced antigenic competition in the nasal mucosa compared to the peroral and intragastric route may also present other advantages of *Bordetella* as a live attenuated vector.

In accordance with the present invention, the genetically modified *Bordetella* may be used as a component of a vaccine.

Bordetella having type III secretion mutants in accordance with the present invention which do not secrete type III secretion factors are also useful in identifying proteins and cloning DNA sequences encoding secreted factors. Factors can be cloned by their presence in the supernatant of cultured wild type bacteria and their absence from the supernatant of cultured modified bacteria. For example, wild type and modified bacteria may be separately grown in Stainer-Scholte medium at 37°C. Culture supernatants may then be precipitated (with TCA, or the like) separated by SDS-PAGE, and stained (with Coomassie blue, or the like). Polypeptides present in the wild type but absent from the modified bacterial supernatant may then be electroblotted (onto PVDF paper, for example) and their

amino-terminal sequences determined (by Edman degradation, or the like). A *Bordetella* genomic or cDNA library, or the like, may then be screened to obtain isolated DNA sequences encoding type III secretion secreted factors, or PCR may be used to amplify and isolate the sequence, which may then be subcloned.

Alternatively, species A (mice for example) may be immunized with supernatant from wild type bacteria, while species B (rabbits, for example) may be immunized with supernatant from modified bacteria. In an expression screen of *Bordetella*, filters may be blocked with the serum from species B, then hybridized with the serum of species A. The filters are then incubated with labeled antibody specific for species A antibodies. As the clones producing products secreted by both wild type and mutant bacteria will have been blocked by antibodies present in the serum from the animals immunized with the mutant bacteria (species B), the antibodies present in the serum from species A will only bind clones producing products secreted by the type III secretion, which will not have been blocked. The clones may then be isolated and the DNA sequences encoding the secreted factor(s) subcloned into an appropriate vector. Alternatively, a genetic strategy may be used. For example, Tn*cya*A mutagenesis to detect genes encoding polypeptides translocated into host cells, and the use of screens relying on regulatory factors specific to Type III gene expression to find co-regulated genes. Other like strategies may be used with the type III secretion mutants to clone DNA sequences encoding polypeptides and proteins secreted by the type III secretion system.

Another aspect of the present invention is a *Bordetella* type III secretion effector or combination of effectors, useful for sequestration of *NF-κB* in the cytoplasm. The effector, or effectors are also useful for preventing signal transduction to progress such that *NF-κB* activation and nuclear localization does not occur. The effector or effectors are also useful in preventing *NF-κB* from activating gene transcription, including preventing *NF-κB* from activating expression of cytokine and/or anti-apoptotic genes.

Another aspect of the present invention includes a *Bordetella* type III secretion effector or combination of effectors, useful for *in vivo* decreasing inflammation and increase apoptosis of inflammatory cells. These components find utility as anti-inflammatory agents, particularly at mucosal sites. Their use is particularly effective in the airways and lungs.

Another aspect of the present invention includes a *Bordetella* type III secretion effector or combination of effectors, with an in vivo tyrosine phosphatase activity.

The type III secretion system is also useful for testing compounds for antimicrobial agents. Agents that inhibit type III secretion of wild type *Bordetella* are effective in preventing the inhibition of inflammation, thus permitting the immune system of the host organism to clear the *Bordetella* infection.

Also provided by the invention are vectors that comprise the nucleic acid sequences described above. Examples of suitable vectors include but are not limited to retroviruses such as Moloney murine leukemia virus (MoMuLV); papovaviruses such as JC, SV40, polyoma, adenoviruses, Epstein-BalT Virus (EBV); papilloma viruses, e.g. bovine papilloma virus type I (BPV); vaccinia and poliovirus and other human and animal viruses.

Adenoviruses have several properties that make them attractive as cloning vehicles (Bachettis et al.: *Transfer of gene for thymidine kinase-deficient human cells by purified herpes simplex viral DNA*, PNAS USA 74:1590 (1977); Berkner, K.L., *Development of adenovirus vectors for expression of heterologous genes*, Biotechniques, 6:616 (1988); Ghosh-Choudhury G, et al., Human adenovirus cloning vectors based on infectious bacterial plasmids, Gene; 50:161 (1986); Hag-Ahmand Y, et al., *Development of a helper-independent human adenovirus vector and its use in the transfer of the herpes simplex virus thymidine kinase gene*, J Virol 57:257 (1986); Rosenfeld K et al., *Adenovirus-mediated transfer of a recombinant al-antitrypsin gene to the lung epithelium in vivo*, Science 252:431 (1991)).

For example, adenoviruses possess an intermediate sized genome that replicates in cellular nuclei; many serotypes are clinically innocuous; adenovirus genomes appear to be stable despite insertion of foreign genes; foreign genes appear to be maintained without loss or rearrangement; and adenoviruses can be used as high level transient expression vectors with an expression period up to 4 weeks to several months. Extensive biochemical and genetic studies suggest that it is possible to substitute up to 77.5 kb of heterologous sequences for native adenovirus sequences generating viable, conditional, helper-independent vectors (Kaufman R.J.; *Identification of the component necessary for adenovirus translational control and their utilization in cDNA expression vectors*, PNAS USA 82:689 (1985)).

Another vector is AAV. AAV is a small human parvovirus with a single stranded DNA genome of approximately 5 kb. This virus can be propagated as an integrated provirus in several human cell types. AAV vectors have several advantage for human gene therapy. For example, they are trophic for human cells but can also infect other mammalian cells; (2) no disease has been associated with AAV in humans or other animals; (3) integrated AAV genomes appear stable in their host cells; (4) there is no evidence that integration of AAV

alters expression of host genes or promoters or promotes their rearrangement; (5) introduce genes can be rescued from the host cell by infection with a helper virus such as adenovirus.

HSV-1 vector system facilitates introduction of virtually any gene into non-mitotic cells (Geller et al., An efficient deletion mutant packaging system for a defective herpes simplex virus vectors: Potential applications to human gene therapy and neuronal physiology, PNAS USA 87:8950 (1990)).

Another vector for mammalian gene transfer is the bovine papilloma virus-based vector (Sarver N, et al., *Bovine papilloma virus DNA: A novel eukaryotic cloning vector*, Mol Cell Biol 1:486 (1981)).

Vaccinia and other poxvirus-based vectors provide a mammalian gene transfer system. Vaccinia virus is a large double-stranded DNA virus of 120 kilodaltons (kd) genomic size (Panicali D, et al., *Construction of poxvirus as cloning vectors: Insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccine virus*, Proc Nad Acad Sci USA 79:4927 (1982); Smith et al., *Infectious vaccinia virus recombinants that express hepatitis B virus surface antigens*, Nature 302:490 (1983).)

Retroviruses are packages designed to insert viral genes into host cells (Guild B, et al., *Development of retrovirus vectors useful for expressing genes in cultured murine embryonic cells and hematopoietic cells in vivo*, J Virol 62:795 (1988); Hock RA, et al., *Retrovirus mediated transfer and expression of drug resistance genes in human hemopoietic progenitor cells*, Nature 320:275 (1986)).

The basic retrovirus consists of two identical strands of RNA packaged in a proviral protein. The core surrounded by a protective coat called the envelope, which is derived from the membrane of the previous host but modified with glycoproteins contributed by the virus.

Further, microorganisms are provided that are transformed by vectors including DNA sequences encoding constituents of the secretory system of the present invention. In accordance with the practice of the invention, the microorganism is *Bordetella*, *e.g.*, *Bordetella* selected from the group including, but not limited to, *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. The invention further provides host cells infected with the microorganisms of the invention.

Additionally, vaccines for protecting an animal against a disease are provided. For example, the vaccine can induce a cytotoxic T lymphocyte (CTL) mediated immune response. In one embodiment of the invention, the vaccine comprises a sufficient amount of microorganisms of the invention. The microorganisms of the invention have been transformed by the vectors of the invention. In accordance with the practice of the invention,

a vaccine can further comprise a sufficient amount of one or more additional antigenic components for protecting an animal against disease caused by one or more other pathogenic microorganism, cells or viruses. Examples of suitable antigenic components include but are not limited to one or more of inactivated leptospira canicola, inactivated leptospire icterhemmorhagiae, modified canine distemper virus, modified canine adenovirus type 2, modified canine parainfluenza virus and modified canine parvovirus.

The invention further provides hybrid nucleic acid sequences. These hybrid nucleic acid sequences encode heterologous gene products and comprise nucleic acid sequences encoding members of a type III secretion system for *Bordetella* joined to a transgene or multiple transgenes. These heterologous gene products can stimulate host immunity, e.g., by a CTL mediated immune response, and serve as a vaccine for *Bordetella* or non-*Bordetella* infection. In one embodiment, the transgene encodes an immunogenic protein from any species. Examples of suitable immunogenic proteins include but are not limited to a pilinic subunit, pertussis toxin or subunits thereof, filamentous hemagglutinin, adenylate cyclase and the protein 69K.

In one embodiment, the hybrid nucleic acid sequence includes, however is not necessarily limited to bscV, bcr3, bopN, bsp22, bcrH1, bopD, bopB, bcrH2, bcr4, bscl, bscJ, bscK, bscL, bscN and bscO having the sequences described above and a transgene.

Examples of transgenes include suicide genes and genes that show late cell development. For example, suicide result in a protein or agent that inhibits cell growth or tumor cell death. Suicide genes include genes encoding enzymes, oncogenes, tumor suppressor genes, genes encoding toxins, genes encoding cytokines, or a gene encoding oncostatin. The purpose of the transgene is to inhibit the growth of or kill cells, of interest or produce cytokines or other cytotoxic agents which directly or indirectly inhibit the growth of or kill the cell of interest.

Suitable enzymes include thymidine kinase (TK), xanthine-guanine phosphoribosyltransferase (GPT) gene from *E. coli* or *E. coli* cytosine deaminase (CD), or hypoxanthine phosphoribosyl transferase (HPRT).

Suitable oncogenes and tumor suppressor genes include neu, EGF, ras (including H, K, and N ras), p53, Retinoblastoma tumor suppressor gene (Rb), Wilm's Tumor Gene Product, Phosphotyrosine Phosphatase (PTPase),, and nm23. Suitable toxins include *Pseudomonas* exotoxin A and S; diphtheria toxin (DT); *E. coli* LT toxins, Shiga toxin, Shiga-like toxins (SLT-1, -2), ricin, abrin, supporin, and gelonin.

Suitable cytokines include interferons, GM-CSF interleukins, tumor necrosis factor (TNF) (Wong G, et al., *Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins*, Science 228:810 (1985)); W09323034 (1993); Horisberger MA, et al., *Cloning and sequence analyses of cDNAs for interferon- and virus-induced human Mx proteins reveal that they contain putative guanine nucleotide-binding sites: functional study of the corresponding gene promoter*, Journal of Virolo 64(3):1171-81 (1990); Li YP et al., Proinflammatory cytokines tumor necrosis factor-alpha and IL-6, but not IL-1, down-regulate the osteocalcin gene promoter, Journal of Immunology 148(3):788-94 (1992); Pizarro TT, et al., *Induction of TNF alpha and TNF beta gene expression in rat cardiac transplants during allograft rejection*, Transplantation 56(2):399-404 (1993)). Breviario F, et al., *Interleukin-1-inducible genes in endothelial cells. Cloning of a new gene related to C-reactive protein and serum amyloid P component.* Journal of Biologic Chemistry, 267(31):22190-7 (1992); Espinoza-Delgado I, et al., *Regulation of IL-2 receptor subunit genes in human monocytes. Differential effects of IL-2 and FFN-gamma*, Journal of Immunology 149(9):2961-8 (1992); Algate PA, et al., *Regulation of the interleukin-3 (IL-3) receptor by IL-3 in the fetal liver-derived FL5.12 cell line*, Blood 83(9)-2459-68 (1994); Cluitmans FH, et al., *IL-4 down-regulates IL-2-, IL-3-, and GM-CSF-induced cytokine gene expression in peripheral blood monocytes*, Annals of Hematology, 68(6):293-8 (1994); Lagoo, AS, et al., *IL-2, IL-4, and IFN-gamma gene expression versus secretion in superantigen-activated T cells. Distinct requirement for costimulatory signals through adhesion molecules*, Journal of Immunology 152(4):1641-52 (1994); Martinez OK et al., *IL-2 and IL-5 gene expression in response to alloantigen in liver allograft recipients and in vitro*, Transplantation 55(5):1159-66 (1993); Pang G, et al., *GM-CSF, IL-1 alpha, IL-1 beta, IL-6, IL-8, IL-10, ICAM-1 and VCAM-1 gene expression and cytokine production in human duodenal fibroblasts stimulated with lipopolysaccharide, IL-1 alpha and TNF-alpha*, Clinical and Experimental Immunology 96(3):437-43 (1994); Ulich TR, et al., *Endotoxin-induced cytokine gene expression in vivo. III. IL-6 mRNA and serum protein expression and the in vivo hematologic effects of IL-6*, Journal of Immunology 146(7):2316-23 (1991); Mauviel A, et al., *Leukoregulin, a T cell-derived cytokine, induces IL-8 gene expression and secretion in human skin fibroblasts. Demonstration and secretion in human skin fibroblasts. Demonstration of enhanced NF-kappa B binding and NF-kappa B-driven promoter activity.*, Journal of Immunology 149(9):2969-76 (1992)).

Growth factors include Transforming Growth Factor-α (TGFα) and β (TGFβ), cytokine colony stimulating factors (Shimane M, et al., *Molecular cloning and*

characterization of G-CSF induced gene cDNA, Biochemical and Biophysical Research Communications 199(1):26-32 (1994); Kay AB, et al., *Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects*, Journal of Experimental Medicine 173(3):775-8 (1991); de Wit H, et al., *Differential regulation of M-CSF and IL-6 gene expression in monocytic cells*, British Journal of Haematology 86(2):259-64 (1994); Sprecher E, et al., Detection of IL- I beta, TNF-alpha, and IL-6 gene transcription by the polymerase chain reaction in keratinocytes, Langerhans cells and peritoneal exudate cells during infection with herpes simplex virus-1, Archives of Virology 126(1-4):253-69 (1992)).

Preferred vectors for use in the methods of the present invention are viral including adenoviruses, retroviral, vectors, adeno-associated viral (AAV) vectors. However, other vectors generally known in the art may be used.

The invention also provides recombinant toxin antigens encoded by any of the hybrid nucleic acid sequences described above. Further, the invention provides vaccine compositions comprising pharmaceutically acceptable carriers or diluents in combination with any of the recombinant toxin antigens of the invention. In one embodiment, the vaccine composition further comprises an antigen adjuvant.

The invention additionally provides antibodies or derivatives thereof which recognize and bind a protein of the type III secretion system of the invention derived from type III secretion system of *Bordetella*.

The invention provides methods for protecting an animal (e.g., a dog, cat, pig, or cow) against disease. In one embodiment a method which comprises administering to the animal a vaccine. The vaccine comprises a microorganism of the invention in an amount sufficient to protect the animal. Administration of the vaccine can be effected by parenteral injection, intranasal administration, intrapharyngeal administration, or topical administration. Other means of administration are possible.

In accordance with the practice of the invention, the animal can be a dog and protection is directed to prevention of kennel cough. In this case, administration of the vaccine can be effected by intrapharyngeal application. Other administration means are possible.

Alternatively, the animal is a swine and protection is directed to prevention of atrophic rhinitis and turbinate atrophy. In this case, administration can be effected by intranasal application. Other administration means are possible.

In accordance with the practice of the method of the invention, the amount of the microorganism to be administered is at least one microorganism per administration. The amount of the microorganism can be in a range of 1 microorganism to 100 million microorganisms per administration.

The invention also provides a method for protecting an animal against disease which comprises administering to the animal a vaccine composition comprising a pharmaceutically acceptable carrier or diluent in combination with any of the recombinant toxin antigen of the invention.

Additionally, the invention provides methods for expressing a heterologous gene product from a *Bordetella* strain. The method comprises attaching a transgene to a nucleic acid sequence of the invention so as to produce a hybrid gene. Further, the method provides introducing the hybrid gene into the *Bordetella* strain to form a viable transformed *Bordetella* strain and culturing the transformed *Bordetella* strain to effect expression of the heterologous gene product. In one embodiment, expression of the heterologous gene product is constitutive, or regulated.

The invention also provides methods for the diagnosis of diseases associated with a *Bordetella* strain in a subject. This method comprises obtaining a specimen, *e.g.*, of nasopharyngeal secretions, from the subject and detecting the presence of a nucleic acid sequence of the invention. After detection is effected, the method provides quantitatively determining the number of nucleic acid sequences so present. In accordance with the practice of the invention quantitatively determining the number of nucleic acid sequences can be achieved by comparing the number of cells so detected to the amount in a sample from a normal subject. The presence of a measurable different amount indicating the presence of the disease.

Alternatively, the number of nucleic acid sequences can be achieved by comparing the number of cells so detected to the amount in a sample from the same subject at a different point in time so that a difference can be determined. The difference in time being indicative of the state of the disease.

In accordance with the practice of the invention the subject includes, but is not limited to, a dog, cat, pig, cow.

The invention further provides methods for detecting the presence of nucleic acid sequences encoding a proteins of the type III secretion system for *Bordetella* in a sample.

In one embodiment, the invention comprises contacting a protein encoded by a nucleic acid with the antibody of the invention thereby forming a detectable complex. The

presence of the complex in the sample is indicative of the presence of the nucleic acid sequence. In accordance with the practice of the invention, the antibody can be labeled so as to directly or indirectly produce a detectable signal. The label includes but is not limited to a compound such as a radiolabel, an enzyme, a chromophore and a fluorescer.

Further, in another embodiment, the invention comprises contacting the sample with any of the nucleic acid sequence of the invention and detecting the binding of the nucleic acid to a constituent in the sample thereby forming a complex. The presence of the complex being indicative of the nucleic acid encoding any of the bscV, bcr3, bopN, bsp22, bcrH1, bopD, bopB, bcrH2, bcr4, bscl, bscJ, bscK, bscL, bscN and bscO protein in the sample. In accordance with the practice of the invention, the constituent can be a DNA or RNA. Further, the sample can be a tissue or biological fluid sample. Examples of biological fluids include but is not limited to urine and blood sera.

In accordance with the practice of the invention, the nucleic acid sequence can be labeled so as to directly or indirectly produce a detectable signal. Typically, the label is a compound selected from the group consisting of a radiolabel, an enzyme, a chromophore and a fluorescer. Other compounds are possible.

The invention further provides a method for producing a *Bordetella* bacteria having an inactivated secretion system. This method comprises genetically modifying the *Bordetella* bacteria by removing any of the type III secretion genes, e.g., any or all of the nucleic acid sequences of invention. Alternatively, the method comprises genetically modifying the *Bordetella* bacteria by modifying any of the type III secretion genes so as to inhibit secretion or transfer of the gene product to the host. The genetically modified *Bordetella* having an inactivated secretion system so produced can be used as an attenuated strains for a vaccine against a *Bordetella* infection.

Further, the invention provides a method for inhibiting infectious *Bordetella* bacteria in a subject by administering the genetically modified *Bordetella* so produced by the method above to a subject. Transient or limited colonization by the modified Bordetella results in protection against infectious bacteria.

The type III system of the invention can be used to deliver macromolecules, *e.g.*, proteins of interest, directly into host cells. There has been no previous report of a type III secretion system in *Bordetella spp.* and all of the previously identified protein toxins synthesized by *Bordetella spp.* do not appear to be secreted by a type III system. The data herein shows that genes of a type III secretion apparatus when expressed, *e.g.*, in *B. bronchiseptica*, is regulated by the BvgAS two-component signal transduction system.

The regulation of type III secretion is useful since secretion of many proteins via this pathway appears to be facilitated by direct contact between bacteria and target cells (Galan and Bliska, 1996). Environmental signals including pH, redox potential, osmolarity, and nutrient levels can also regulate expression of type III system genes (Bajaj et al., 1996; Lee, 1997).

The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.

EXAMPLES

Experimental procedures

Bacterial strains and growth conditions.

B. bronchiseptica strains RB50, RB54 and RB53; *B. pertussis* strains Tohama 1, 18323 and GMT1; ovine isolate of *B. parapertussis* strain H1 have all been previously described (Cotter and Miller, 1994; Martinez de Tejada et al., 1996; Porter et al., 1994). *B. pertussis* strain 17474 was a clinical isolate from Erlangen, Germany and human isolate of *B. parapertussis* strain A168 was from CDC, USA. *B. bronchiseptica* strain FF1 was constructed from RB54 by replacing wild type promoters of *fhaB* and *fhaC* with the *flaA* promoter to ectopically express FHA. All strains were cultured in Stainer-Scholte liquid medium or BG agar as previously described (Cotter and Miller, 1994; Cotter and Miller, 1997; Martinez de Tejada et al., 1996).

Differential display, PCR, RT-PCR, RNA slot blots, DNA sequencing and molecular cloning.

Total RNA was isolated from mid-log bacterial cultures using Trizol reagent (Gibco) according to the manufacturer's protocol. Total RNA was reversed-transcribed into cDNA using 2μg RNA, 200 ng random hexamers and Superscript II (Gibco) as described by manufacturer's protocol. Reaction conditions for differential display/arbitrary-primed PCR were as follows: One-tenth or one-twentieth of the cDNA from each reverse-transcription reaction was combined with 3mM $MgCl_2$, 1 U Taq Polymerase (Promega, Madison WI), 250 μM each of the 4 dNTPs and 20 pmoles of primer in a total volume of 25 μl. A PTC-100 thermal cycler (MJ Research) was used for the reactions. For short primers (10-mers), the cycling parameters were: 45 cycles of (94°C for 1 min, 36°C for 1 min, 72°C for 2 min) and then 72°C for 5 min. For longer primers (>17 mers): 4 cycles of (94°C for 5 min, 40°C for 5

min and 72°C for 5 min) were followed by 30 cycles of (94°C for 1 min, 55°C for 1 min and 72°C for 2 min) and a final incubation at 72°C for 5 min.

PCR products were analyzed on 2% agarose gels containing 0.5 μg/ml ethidium bromide. To determine reproducibility, reactions with different concentrations of cDNA were run in duplicates. Specific bands of interest were isolated with Geneclean kit (Biol0l), reamplified under the same PCR conditions, and then cloned using the TA cloning kit (Invitrogen).

For PCR with specific primer pairs, conditions were as follows: 2 mM $MgCl_2$, 1 U Taq Polymerase (Promega, Madison WI), 200 μM each of the 4 dNTPs, 1 μM of each primer and 5% DMSO. For genomic DNA templates, a single colony of bacterial culture on a plate was picked and directly mixed into the reaction solution. For cDNA templates, one-twentieth of each reverse-transcription reaction was used. Cycling parameters were: 25 cycles of (94°C for 1 min, 55° or 50°C for 1 min and 72°C for 1 min) and a final incubation at 72°C for 5 min. Sequence of primers used are shown in Table 2.

RNA slot blots were performed as previously described (Cotter and Miller, 1997). Probes used were: a 500 bp EcoRI-StyI fragment of *recA*, a 450 bp *BglII-BamHI* fragment of *fhaB*, a 500 bp PCR product of *flaA* using primers BA04 and BA01 1, and a 420 bp PCR product of *bscN* using primers W3 and W4. All recombinant DNA techniques were performed as described in standard protocols (Sambrook et al., 1989).

Bacterial conjugations, allelic exchanges, plasmid rescues and construction of in-frame deletion.

Allelic exchanges were performed using suicide vectors pEG7 or pEGBR (Akerley et al., 1995; Cotter and Miller, 1997; Martinez de Tejada et al., 1996). DNA fragments used for homologous recombinations were subcloned into the vectors and then transformed into *E. coli* SM10 for mating to *B. bronchiseptica*. Matings, selection for gentamicin or kanamycin resistant co-integrants and counter-selection against sucrose sensitivity for second recombination events were done as described (Akerley et al., 1995; Cotter and Miller, 1997; Martinez de Tejada et al., 1996).

DNA flanking original fragment of *bscN* (from arbitrary-primed PCR) was isolated as follows: the 420 bp PCR fragment was subcloned into pEG7 and the resulting suicide plasmid introduced into RB50. Genomic DNA from gentamicin resistant colonies (containing integrated plasmid by homologous recombination into the *bscN* gene) was digested with *NsiI* (one of several restriction enzymes used which does not cut within pEG7),

self-ligated, transformed into E. coli XL1-Blue and selected by ampicillin resistance. The rescued plasmid containing extra 4 kb of DNA was restriction mapped and fragments were subcloned into pBluescript for DNA sequencing on both strands. The assembled sequence was analyzed for ORFs and searched for homologous sequences in the database using BLAST (NCBI) and sequence alignments were performed with ALIGN in the FASTA program (U. Virginia).

For construction of the in-frame deletion in *bscN*, two PCR fragments using primers W1+W2, which amplify a 350 bp fragment (from codon no. 54 to codon no. 170 of the *bscN* ORF) and primers W3+W4, which amplify a 420 bp fragment (from codon 262 to 400) were ligated by overlapping PCR, using overlapping regions between W2 and W3, in the presence of primers W1 and W4. Pfu polymerase (Stratagene) was used for these PCRs. The resultant 770 bp fragment was sequenced to ensure maintenance of the reading frame and then subcloned into pEGBR. The resulting suicide vector was introduced into RB50 and two recombination events were selected for (first by kanamycin resistance and then by sucrose resistance). Resulting colonies were screened by PCR with primers W1 and W4 which give a 770 bp product from the genome of the deletion strain WD3 but a 1050 bp product from the wild type. For construction of the transcriptional *LacZ* fusion with *bscN*, the 420 bp PCR product from W3+W4 was subcloned into the suicide vector pEGZ (Martinez de Tejada et al., 1996), integrated into RB50 genome by homologous recombination, and selected by gentamicin resistance.

For construction of the in-frame deletion in *bsp22*, two PCR fragments using primers
D21 (5'GCGGATCCAGTTTTGCCTGCGCGTCG3') and
D22 (5'AACTCCGAGATCAATGGTCATG3')
which amplify a 490 bp fragment (from upstream of *bsp22* ORF to including first 7 codons) and primers
D23 (5'ATGACCATTGATCTCGGAGTTAACAGTTCCATCACCAACAAC3') and
D24 (5'GCGGATCCAACCCCTGCAAGCTGCCC3')
which amplify a 460 bp fragment (from the last 9 codons to downstream of *bsp22* ORF) were ligated by overlapping PCR, using overlapping regions between D22 and D23, in the presence of primers D21 and D24. The resultant 930 bp fragment was sequenced to ensure maintenance of the reading frame and then subcloned into pEGBR. The resulting suicide vector was introduced into RB50 (via conjugation with transformed *E. coli* SM10 strains) and two recombination events were selected for (first by kanamycin resistance and then by sucrose resistance). Resulting colonies were screened by PCR with primers D21 and D24

that gave a 930 bp product from the genome of the deletion strain D218 but a 1490 bp product from the wild type. Functional deletion of Bsp22 in D218 was confirmed by immunoblots. For complementation of D218, an 850 bp fragment consisting of the entire ORF of *bsp22* was obtained by PCR with the primers

LB25K (5'ACTGGTACCTCGGAGAAGGAACCATTTGCCTAC3') and

B2FF3B (5'CTAGGATCCGCGGCACGCATGGATTGG3')

which was subcloned into KpnI and BamHI sites of the broad host range plasmid pBBR1MCS (Kovach, et al., *Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes*, Gene 166(1):175-6 (1995)) to create the plasmid pLB2. pLB2 was transformed into *E. coli* stain SM10 which in turn was conjugated to D218 and resultant ex-conjugants selected by antibiotic resistance.

SDS-PAGE, Western immunoblots and ELISA, protein sequencing, antibody production and β-galaclosidase assays.

Protein analysis on SDS-PAGE and immunoblots were performed as previously described (Cotter and Miller, 1997; Martinez de Tejada et al., 1996). Proteins from 15 hour culture supernatants were first precipitated with 10% trichloroacetic acid (TCA) for 4 hours on ice. 10 ml equivalent of supernatant was used for each lane loaded for detection by Coomassie staining while 1 ml equivalents were used for immunoblots. Total cellular proteins from L2 cell cultures were obtained by solubilizing cells on 6-well tissue culture plates with 300 µl of protein sample buffer and then scraped off and boiled for 5 minutes. One-tenth of that was loaded on each lane. Sera from rabbits, rats and mice previously infected with *B. bronchiseptica* for 1 to 2 months were diluted 1:2500 for immunoblotting. Anti-phosphotyrosine monoclonal antibody from mouse was clone PT-66 from Sigma and it was used at 1:2000 dilution for immunoblots. All detections of immunoblots were done with enhanced chemiluminescence (Amersham). P-galactosidase assays were performed as previously described (Martinez de Tejada et al., 1996).

For protein amino-terminal sequence determination, proteins separated by SDS-PAGE were electroblotted onto PVDF paper and stained with Coomassie Blue. A GST fusion of the entire ORF of bsp22 was constructed and purified with standard protocols using pGEX2T vector (Pharmacia). 8 week old BALB/c mice were injected i.p. with 100 µg of the GST-fusion protein in a 1:9 emulsion with complete Freund's adjuvant on days 0, 14, 28, 35 and 42 to induce antibody and ascites production. Ascites from the mice were collected on days 35, 42 and 49, and tested for specificity to antigens with immunoblots. Rabbit and

mouse antisera were diluted 1:2500 for immunoblotting. All detections of immunoblots were done with enhanced chemiluminescence (Amersham).

Mammalian cell cultures, cytotoxicity assays, apoptosis assays and immunofluorescent studies.

L2 rat lung epithelial cell line (ATCC) was cultured in F12K medium supplemented with 10% fetal bovine serum (FBS) while J774 and RAW macrophage-like cell lines were cultured in DME with 10% FBS. L2 cells were grown to 70% confluency for cytotoxicity assays and 95% confluency for phosphotyrosine assay. Bacterial infections of the cell cultures were done with multiplicity of infection (MOI) ranging from 10 (for macrophage cell lines) to 500 (for L2 cells) and bacterial suspensions were centrifuged onto the adherent cells at 500Xg for all the assays. For L2 cytotoxicity assays, at the end of incubations, the cells were washed three times with Hanks Balanced Salt Solution (HBSS), fixed in methanol and stained with Giemsa stain for 30 minutes before observations under a phase-contrast microscope. Cytotoxicity assays from the macrophage cell lines were performed using the Cytotox96 kit (Promega) as per manufacturer's protocol.

Apoptosis assays were performed using the *in situ* cytotoxicity assay kit based on a fluorescent TUNEL assay (Boehringer Mannheim) according to manufacturer's protocol. Cytotoxicity assays for the J774A.1 cell line were performed using the Cytotox96 kit (Promega) according to manufacturer's protocol. Immunofluorescent labeling of L2 cells were performed as follows: cells were fixed for 30 minutes in 4% paraformaldehyde and permeabilized with 0.1% TritonX-100 in PBS for 10 minutes. Fixed samples were pre-incubated for 30 minutes in 1% BSA/Hanks balanced salt solution (HBSS) with 1:100 dilution of normal goat serum, followed by 60 minutes in 1:200 dilution of primary antibody (against p65 subunit of NF-κB, sc-372, Santa Cruz Biotechnology) in the same buffer. They were then washed 3 times in 1% BSA/HBSS and then incubated with 1:200 dilution of Alexa568 tagged goat anti-rabbit antibody (Molecular Probes) for 30 minutes. Cells were then washed 3 times in PBS and observed under epifluorescent microscopy.

Experimental animals.

Infection of 4 week old female Wistar rats with either RB50 or WD3 was done as previously described (Akerley et al., 1995; Cotter and Miller, 1997; Martinez de Tejada et al., 1996). 1000 cfu of each strain in a 5 µl volume was inoculated intra-nasally. Statistical significance of differences in colonization was determined by unpaired t-test comparisons. Statistical significance of the differences in survival time was determined by Logrank

(Mantel-Cox) comparisons. All experiments were in accordance with guidelines and protocols approved by UCLA Animal Research Committee.

Identification and cloning of a yscN *homologue from* B. bronchiseptica.

The technique of DD-PCR was used with arbitrary primers to identify genes that are differentially expressed under Bvg^+ or Bvg^- growth conditions in *B. bronchiseptica in vitro*. Total RNA was isolated from *B. bronchiseptica* strain RB50 grown under non-modulating (Bvg^+) or modulating (Bvg^-) conditions and reverse-transcribed to cDNA. The cDNAs were used as templates for PCR amplification using arbitrary primers ranging from 10 to 35 nucleotides in length. Several primers amplified distinct fragments that were specific to the Bvg^+ phase. One of the primers, KB4, produced a 480 bp band from Bvg^+ phase cDNA only (Fig. 1, lanes 1 and 2). This band was cloned, sequenced and found to correspond exactly to an internal sequence of *cyaA*, the Bvg-activated gene encoding adenylate cyclase toxin/hemolysin. Another primer, ML2, produced a 420 bp fragment specifically from Bvg^+ phase, but not Bvg^- phase cDNA (Fig. 1, lanes 5 to 8). The DNA and the predicted amino acid sequence of this fragment showed a high degree of similarity to the 3' end of the ORF for the *yscN* gene from *Yersinia spp.* (Bergman et al., 1994, Woestyn et al., 1994). The YscN gene product is postulated to hydrolyze ATP to provide energy for secretion of Yops (*Yersinia* outer proteins) via the type III secretion system. There has been no previous report of type III secretion genes in *Bordetella spp.* The 420 bp RT-PCR fragment was used to clone the complete ORF and flanking genes by plasmid rescue (described in Experimental procedures). The 420 bp fragment lies within an ORF encoding a predicted 48 kD protein with a 64% amino acid identity to YscN from *Yersinia* (Fig. 2a). This *B. bronchiseptica* protein was designated BscN.

BscN contains two conserved Walker Boxes (Walker et al., 1982), suggesting it can bind and hydrolyze ATP. Fourteen other ORFs flank *bscN* (Fig. 3). Thirteen of these ORFs show amino acid sequence similarities to proteins in *Yersinia spp.* which comprise part of the Ysc type III secretion apparatus (Allaoui et al., 1995; Bergman et al., 1994), and also the corresponding Psc homologues in *Pseudomonas aeruginosa* (Yahr et al., 1996). The homologous ORFs were designated in *B. bronchiseptica* as bscV, bcr3, bopN, bcrH1, bopD, bopB, bcrH2, bcr4, bscl, bscJ, bscK, bscL, bscN and bscO. The nucleotide sequence of a 4.6 kb fragment containing some of these genes have been deposited in Genbank under the accession number AF049488 (incorporated herein by reference in their entirety). A 13500bp sequence corresponding to the 15 ORF and flanking sequences is presented in figure 18. The organization of these ORFs suggests that they may be transcribed as an operon.

BscN *expression is positively regulated by BvgAS.*

Three assays were used to investigate the role of BvgAS in the control of *bscN* expression: RT-PCR, slot blot hybridizations of total RNA and *lacZ* transcriptional fusion analysis. Figure 4a shows results of RT-PCR using various primer pairs on genomic or cDNA templates prepared from RB50 grown under Bvg^+ or Bvg^- phase conditions. Primers specific for the filamentous hemagglutinin (FHA) structural gene (*fhaB*), a Bvg^+ phase factor, gave rise to a PCR product of the correct size only from cDNA made from Bvg^+ phase RNA. Primers specific for flagellin (*fhaA*), a Bvg^- phase gene, gave rise to a PCR product only from cDNA made from Bvg^- phase RNA. These controls confirmed that the RNAs from Bvg^+ and Bvg^- phase grown RB50 contain phase-specific transcripts. Mock reactions, which did not contain reverse transcriptase in the RT reactions, did not give rise to PCR products, confirming negligible genomic DNA contamination in the RNA preparations. The two pairs of primers specific for bscN (W1+W2, which amplify a 350 bp region in the 5' end; and W3+W4, which amplify a 420 bp region in the 3' end) generated products of the expected sizes only from cDNAs derived from Bvg^+ phase RNA. cDNAs were also prepared from Bvg^+ and Bvg^- phase-locked derivatives of RB50, RB53 and RB54, respectively. Only cDNAs prepared from RB53 RNA generated PCR products of the expected sizes with primers specific for *bscN* (Fig. 4b). RT-PCR with primers specific for the *bscIJKL* loci produced the same results, showing that they were transcribed only in the Bvg^+ phase.

To confirm the results from RT-PCR, a DNA probe specific for 420 bp of the 3' end of *bscN* was used to hybridize to total RNA on slot blots (Fig. 4c). The control probes for transcripts of *fhaB* (a Bvg^+ phase locus), *flaA* (a Bvg^- phase locus) and *recA* (not regulated by Bvg) showed that the RNA preparations were quantitatively loaded and Bvg phase specific. The probe for *bscN* showed a much higher degree of hybridization to the RNA derived from Bvg^+ phase compared to Bvg^- phase grown cultures. Finally, a strain of RB50 containing a transcriptional fusion of the *lacZ* gene integrated into the 3' end of the chromosomal *bscN* locus was constructed. Beta-galactosidase assays of this strain grown under Bvg^+ and Bvg^- phase conditions during exponential growth showed a twenty-five-fold induction in Bvg^+ phase (β-galactosidase units of 362±65 in Bvg^+ phase versus 13±5 units in the Bvg^- phase). *bscN* expression is tightly regulated by the *bvg* locus and it is transcribed preferentially in the Bvg^+ phase.

BscN is required for secretion of specific Bvg^+ phase polypeptides.

Absence of a functional *bscN* abolishes type III secretion. An in-frame deletion in the RB50 *bscN* locus was constructed as described above. The resulting strain, designated WD3,

has a deletion that removes both Walker Boxes (see Fig. 2a) and should render the BscN protein functionally inactive, based on data from similar mutations in *Yersinia spp.* (Woestyn et al., 1994). WD3 shows a similar growth rate in Stainer-Scholte medium compared to that of RB50 (doubling time of 85 minutes for WD3 versus 90 minutes for RB50 at 37°C with aeration).

To determine if BscN is involved in protein secretion in *B. bronchiseptica*, the protein profile of culture supernatants from RB50 and WD3 was compared. WD3 produced and secreted FHA at levels comparable to RB50 based on immunoblots and also showed the same degree of hemolysis on BG blood agar, suggesting the secretion of FHA and AC/HLY were not noticeably affected by the *bscN* mutation. Total cellular protein preparations from RB50 and WD3 showed very similar profiles on SDS-PAGE based on Coomassie staining and on immunoblots with post-infection sera against *B. bronchiseptica*. However, comparison of Coomassie stained proteins from supernatants of 15 hour cultures of RB50 and WD3 grown under Bvg^+ phase conditions revealed several polypeptides in the culture supernatants from RB50 but not WD3 (Fig. 5, lane 1 and 2, open arrows).

Immunoblot analysis of concentrated supernatant with sera from a rabbit, rat and mouse previously infected with RB50 also identified proteins secreted by RB50 but not by WD3 (Fig. 5, lanes 3 to 9, solid arrows). These proteins are secreted only in the Bvg^+ phase as none of them were detected in the culture supernatant of a Bvg^- phase-locked strain, RB54 (lane 5). Interestingly, the most prominent bands observed on immunoblots did not correspond to the most significant bands detected by the Coomassie staining, with the exception of the 22kD band. Although the antisera were from different mammalian species, they all recognized the same proteins that were differentially secreted from RB50 and WD3. When antisera from animals infected with WD3 were used as probes, none of the differentially secreted proteins were detected (Fig. 5, lanes 10 to 13). This correlates with the observation that the proteins were secreted from RB50 and not WD3. These results indicate that the in-frame deletion in *bscN* leads to a significant decrease of a subset of Bvg^+ phase polypeptides in the culture supernatants. Detection of these bscN-dependent secreted proteins by antisera from RB50 infected animals indicates that they are antigenic and expressed *in vivo*.

At least 9 polypeptides are secreted by type III secretion apparatus in *B. bronchiseptica*.

In-frame deletion of bscN *leads to decreased cytotoxicity of* B. bronchiseptica *to mammalian cells* in vitro.

B. bronchiseptica adheres to a variety of mammalian cells in culture including L2 rat lung epithelial cells and J774 macrophage-like cells. In both cases, binding is Bvg^+ phase dependent. After prolonged binding of RB50 to L2 cells (2 hours or more), it was observed that the L2 cells began to show signs of toxicity: the cytoplasm shrinks, cells round up and detach from the culture plates (Fig. 6c). The *bscN* deletion strain WD3, which in the Bvg^+ phase bound to L2 cells with similar efficiency as RB50, did not lead to the same degree of observable toxicity to L2 cells over the same time period (Fig. 6f). A Bvg^- phased-lock strain which has been engineered to ectopically express FHA (strain FF1) but does not express other Bvg^+ phase factors, could bind to L2 cells but did not elicit any signs of cytotoxicity for over 2 hours (Fig. 6d). Incubation of L2 cells with only the supernatant from cultures of RB50 did not appear to cause cytotoxic effects.

To measure the cytotoxicity of various strains of *B. bronchiseptica* towards macrophage cell lines J774 and RAW, the release of lactate dehydrogenase was measured after a 4 hour incubation with bacteria. WD3 was significantly less cytotoxic to these cells than RB50 (Table 1). Therefore, cytotoxicity of *B. bronchiseptica* towards phagocytic and non-phagocytic mammalian cells *in vitro* depends, at least partially, on a functional BscN.

Wild type B. bronchiseptica, *but not the* bscN *deletion strain, causes tyrosine dephosphorylation of mammalian cell proteins when bound to L2 cells.*

One of the Yops secreted by the type III secretion system in *Yersinia* is YopH which has sequence similarity to mammalian protein-tyrosine phosphatases (PTP) and is injected into host cells upon contact, causing tyrosine dephosphorylation of specific host cell proteins (Andersson et al., 1996, Black and Bliska, 1997; Persson et al., 1997). To determine if *B. bronchiseptica* causes any change in tyrosine-phosphorylation of host cell proteins upon attachment, bacteria were bound to L2 cells for one hour, then total proteins were separated by SDS-PAGE and probed with antibody specific for phosphotyrosine. Figure 7, lane 1 shows that only one protein from *B. bronchiseptica* itself was recognized by the antibody while lane 2 shows a number of tyrosine phosphorylated polypeptides from uninfected L2 cells. Within one hour of binding to L2 cells, wild type Bvg^+ phase RB50 caused the tyrosine dephosphorylation of two or more high molecular weight polypeptides from the L2 cells (lane 3, arrows). This dephosphorylation could be inhibited in the presence of 1 mM vanadate (lane 4), which inhibits PTPs. This dephosphorylation process did not occur following the binding of Bvg^+ phase WD3 to L2 cells (lane 5) or FF1, the Bvg^- phase-locked

strain expressing FHA adhesin (lane 6). Effector proteins secreted by *B. bronchiseptica* into host cells via the Bsc type III secretion system cause tyrosine dephosphoylation of specific host proteins.

B. bronchiseptic*a type III secretion system dependent apoptosis.*

Bvg+ phase *B. bronchiseptica* adhere to a variety of mammalian cells in culture, including rat lung epithelial (L2) cells and J774A.1 and RAW macrophage-like cells. Prolonged binding of RB50 to L2 cells (>2 hours) caused the L2 cells to show marked signs of cytotoxicity. Lactate dehydrogenase release measurements also support the cytotoxicity of RB50 to J774A.1 and RAW cells. None of these cells displayed signs of cytotoxicity when bound by FF1, a Bvg- phase-locked strain that binds the cells by virtue of an ectopically expressed FHA. In all three cell lines, cytotoxicity was shown to be dependent on type III secretion, as WD3 induced less than 4% of the levels of cytotoxicity observed following incubation with RB50.

The cytotoxicity resulting from the activity of the type III secretion system is correlated with apoptosis. Fluorescent TUNEL reagent was used to detect DNA fragmentation, a characteristic of apoptotic cells (Fig. 15). At 30 min. post infection 30% of L2 cells and 60% of J774A.1 cells incubated with RB50 contained strongly labeled nuclei. In contrast, although L2 and J774A.1 cells incubated with WD3 had similar numbers of adherent bacteria, a negligible fraction of nuclei were stained in the TUNEL reaction. DNA fragmentation analysis showed similar results. Thus, wild type *B. bronchiseptica* can induce apoptosis in both phagocytic and non-phagocytic mammalian cells. A type III secretion effector or combination of effectors, is useful for inducing apoptosis in mucosal epithelial and immune cells.

B. bronchiseptic*a type III secretion system dependent NF-κB sequestration.*

NF-κB is a eukaryotic transcription factor that plays a central role in mediating gene expression induced by pathogens and other noxious stimuli. *NF-κB* is activated when it is released from *I-κB*, an inhibitory subunit which masks *NF-κB*'s nuclear localization signals. Upon translocation from the cytoplasm to the nucleus, *NF-κB* functions as a transcriptional activator for a variety of genes including those encoding inflammatory cytokines such as IL-6, IL-8, TNFα and GM-CSF. *NF-κB* has also been shown to play a role in preventing apoptosis, most likely by inducing the expression of "anti-apoptotic genes."

Our experiments using an anti- *NF-κB* antibody and immunofluorescence microscopy showed that type III secreted effector factors alter *NF-κB* activity. (Fig. 17.) In uninfected

L2 cells, *NF-κB* staining was diffuse and evenly spread throughout the cytoplasm. Stimulation by TNFα resulted in intense nuclear staining, indicative of *NF-κB* translocation into the nucleus. In contrast, in cells infected with RB50, anti- *NF-κB* staining was concentrated in discrete cytoplasmic foci, suggesting *NF-κB* was localized in the cytoplasm in large complexes of unknown composition.

The pattern of *NF-κB* staining in WD3 infected cells was the same as in uninfected cells, i.e. diffusely localized throughout the cytoplasm, indicating that the aberrant localization of *NF-κB* seen in RB50 infected cells requires Bsc type III secretion. Furthermore, RB50, but not WD3, inhibits TNFα-mediated translocation of *NF-κB* to the nucleus.

Type III secretion is necessary for long term tracheal colonization in rats.

Wistar rats inoculated with low doses of RB50 (ID_{50}<20) become chronically infected in the nasal cavity and trachea (Akerley et al., 1995; Martinez de Tejada et al., 1996). To determine if WD3 is capable of colonizing the rat respiratory tract, 1000 colony forming units (cfu) of either RB50 or WD3 were inoculated intranasally in a 5 µl droplet into groups of Wistar rats (female, 4 weeks old). After 14 days, similar numbers of cfu were recovered from the nasal septum and trachea of all rats infected with either strain. After 35 days of infection, similar numbers were recovered from the nasal septum of rats infected with either strain. WD3, however, was not found in the trachea of infected animals while all rats infected with RB50 had $>10^5$/centimeter bacteria recovered from the trachea (Fig. 8; see also Fig. 11a and b for other mutants and other species.). Therefore, WD3 appears to be specifically defective in persistence in the trachea, but not in establishing infection or in long-term colonization of the nasal septum. The rather remarkable specificity of the infection phenotype may relate to the fact that the trachea is usually sterile, whereas the nasal cavity is teaming with bacteria. bscN-dependent secretion may therefore be required to evade inducible immune responses that normally protect the lower respiratory tract.

Examination of lung tissue from wild type mice 24 hours post-inoculation with 10^5 cfu of wild type *B. bronchiseptica* delivered in a 50 µl volume showed a modest degree of inflammation. Many of the inflammatory cells that were present appeared to be undergoing apoptosis as indicated by strong nuclear staining with the fluorescent TUNEL reagent. In contrast, lungs from mice infected with WD3 showed a significantly greater degree of inflammation and very few of the cells stained positively with the TUNEL reagent. Fig. 16.

Comparison of bscN *expression in* Bordetella spp.

A primer pair specific for the sequence of *bscN* in *B. bronchiseptica* strain RB50 (W3+W4) were used for PCR amplification of genomic DNA and cDNA from the following strains of *Bordetella*: *B. pertussis* strains Tohama 1, 18323, GMT1 and 17471, *B. parapertussis* strains A168 (human isolate) and H 1 (ovine isolate). All of the strains tested contained the genomic sequence for *bscN* as shown by amplification of an expected 420 bp band from genomic DNA (Fig. 9). Amplification from cDNAs showed that *B. pertussis* strain 18323 and the ovine isolate of *B. parapertussis* produced a detectable transcript from this gene. A very weak signal was detected from *B. pertussis* strain 17471, while Tohama 1 and GMT 1 did not produce any detectable RT-PCR product. Other primers specific for sequences in the *bscJ*, *bscK* and *bscL* loci also gave the same results (i.e. fragments of the expected size from genomic DNA but no products from cDNA). Controls using primers specific for *fhaB* showed that the cDNA preparations were Bvg^+ phase specific. These results indicate that type III secretion genes are present in all the tested *Bordetella* strains. However, under the in vitro conditions used to grow the bacteria for RNA isolation, *B. bronchiseptica* strain RB50, *B. pertussis* strain 18323 and ovine *B. parapertussis* strain H1 showed significant transcription of *bscN* detectable by RT-PCR, while the other *B. pertussis* strains and the human *B. parapertussis* isolate did not.

Generation of genetically engineered, heterologous antigen producing Bordetella.

DNA encoding the heterologous *Pasteurella multocida* antigen, is subcloned between two flanking sequences of *Bordetella* DNA. The flanking sequences are preferably about 500 bps. If the a fusion protein is desired, the flanking sequences are part of the open reading frame in which the antigen will be fused to such that all the sequences are in the same reading frame. The construct is then cloned into a suitable suicide vector, having a selectable marker (antibiotic resistance, for example) and a counter-selectable marker (sucrose sensitivity, for example). pEGBR, for example, may be used. The vector is then transformed into suitable bacteria, E. coli strain SM10, for example. The allelic exchange vector is then transferred into Bordetella by conjugation. *Bordetella* that has received the plasmid and integrated it into the genome at the specific site (determined by the flanking sequences) can be selected by antibiotic resistance encoded by the suicide plasmid. This strain of *Bordetella* is then counter-selected with sucrose to select clones that have undergone a second recombination event to remove the integrated plasmid. Colonies that have the desired antigen DNA sequence integrated into their genome can be identified by PCR with specific primers, southern hybridization or immunoblots with antibodies specific for the antigen, or the like.

For example, an allelic exchange vector with the open reading frame for the desired gene may be inserted in the SnaB1 site between the end of the *B. bronchiseptica* flaA coding region and immediately upstream of the transcription stop site. This construct does not disrupt expression of flaA and does not affect expression of downstream genes. Other constructs using other genes may also be used.

Live attenuated Bordetella *are effective protecting hosts from infection by wild type* B. bronchiseptica.

Eight rats per group were inoculated with 10^4 cfu of either wild type *B. bronchiseptica* (RB50) or the *bscN* mutant (WD3). 35 days later, three rats from each group were sacrificed and colonization levels in the nasal cavities and tracheas were determined. As expected, RB50 was recovered at about 10^5 cfu from each nasal septum and about 10^{4-6} cfu per cm trachea, while WD3 was also recovered at about 10^5 cfu per nasal septum but no WD3 bacteria were recovered from the tracheas. The remaining five rats were challenged with 10^6 cfu of RB50G delivered in a 50µl volume. (RB50G is a derivative of RB50 containing a Gm resistance gene immediately 3' to the *fla*A gene. This strain is indistinguishable from RB50 in its ability to establish persistent infections in rats and mice). A 50µl volume was used for the challenge so that bacteria are delivered to the entire respiratory tract (i.e. the nasal cavity, the trachea and the lungs). Seven days later, the rats were sacrificed and the number of Gm^s and Gm^r bacteria recovered from the nasal septa, larynx, trachea and lungs determined. For rats initially infected with RB50, 2 of the 5 rats contained low numbers (about 10^2) of Gm^r bacteria in their nasal cavities and tracheas while no Gm^r bacteria were recovered from any site in the remaining 3 rats. For animals initially infected with WD3, no Gm^r bacteria were recovered from any site in the respiratory tract from any animal. The number of Gm^s bacteria (RB50 and WD3) recovered from each site was similar to those rats sacrificed before challenge. The attenuated strain, WD3, was therefore superior to wild type *B. bronchiseptica* in its ability to induce protective immunity against wild type *B. bronchiseptica*.

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Claims:

1. A genetically engineered type III secretion system *Bordetella* mutant.

2. The type III secretion system mutant of claim 1 comprising a mutation in a gene encoding a protein or polypeptide selected from the group consisting of core component, effector, accessory factor and combinations thereof.

3. The type III secretion system mutant of claim 2, the mutation being present in *Bordetella* selected from the group consisting of *B. pertussis*, *B. bronchiseptica* and *B. parapertussis*.

4. The type III secretion system mutant of claim 3, the mutation being present in *B. bronchiseptica*.

5. The type III secretion system mutant of claim 2, the mutation being present in gene encoding a protein or polypeptide selected from the group consisting of bscV, bcr3, bopN, bsp22, bcrH1, bopD, bopB, bcrH2, bcr4, bscI, bscJ, bscK, bscL, bscN, bscO and combinations thereof.

6. The type III secretion system mutant of claim 5, the mutation being present in the gene encoding bscN.

7. The type III secretion system mutant of claim 5, the mutation being present in the gene encoding bsp22.

8. The type III secretion system mutant of claim 1, the mutation comprising the deletion of a type III secretion system protein locus.

9. The type III secretion system mutant of claim 1, the mutation comprising an in frame codon deletion of a type III secretion system protein locus.

10. The type III secretion system mutant of claim 1, the mutation comprising a codon substitution of a type III secretion system protein locus.

11. A live, attenuated vaccine component against *Bordetella* comprising a type III secretion system mutant of claim 1.

12. The live, attenuated vaccine component against *Bordetella* of claim 11 comprising a mutation in a gene encoding a protein or polypeptide selected from the group consisting of core component, effector, accessory factor and combinations thereof.

13. The live, attenuated vaccine component against *Bordetella* of claim 11, the mutation being present in a *Bordetella* selected from the group consisting of *B. pertussis*, *B. bronchiseptica* and *B. parapertussis*.

14. The live, attenuated vaccine component against *Bordetella* of claim 13, the mutation being present in *B. bronchiseptica*.

15. The live, attenuated vaccine component against *Bordetella* of claim 12, the mutation being present in gene encoding a protein or polypeptide selected from the group consisting of bscV, bcr3, bopN, bsp22, bcrH1, bopD, bopB, bcrH2, bcr4, bscl, bscJ, bscK, bscL, bscN, bscO and combinations thereof.

16. The live, attenuated vaccine component against *Bordetella* of claim 15, the mutation being present in the gene encoding bscN.

17. The live, attenuated vaccine component against *Bordetella* of claim 15, the mutation being present in the gene encoding bsp22.

18. The live, attenuated vaccine component against *Bordetella* of claim 11, the mutation comprising the deletion of a type III secretion system protein locus.

19. The live, attenuated vaccine component against *Bordetella* of claim 11, the mutation comprising an in frame codon deletion of a type III secretion system protein locus.

20. The live, attenuated vaccine component against *Bordetella* of claim 11, the mutation comprising a codon substitution of a type III secretion system protein locus.

21. A purified nucleic acid comprising a sequence encoding a *Bordetella* type III secretion system component.

22. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 1.

23. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 2.

24. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 3.

25. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 4.

26. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 5.

27. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 6.

28. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 7.

29. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 8.

30. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 9.

31. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 10.

32. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 11.

33. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 12.

34. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 13.

35. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 14.

36. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 15.

37. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 16.

38. The purified nucleic acid of claim 21, the sequence being selected from the group consisting of Seq. ID 1, Seq. ID 2, Seq. ID 3, Seq. ID 4, Seq. ID 5, Seq. ID 6, Seq. ID 7, Seq. ID 8, Seq. ID 9, Seq. ID 10, Seq. ID 11, Seq. ID 12, Seq. ID 13, Seq. ID 14, Seq. ID 15 and Seq. ID 16.

39. An isolated and purified polypeptide encoded by a nucleic acid sequence selected from the group consisting of Seq. ID 2, Seq. ID 3, Seq. ID 4, Seq. ID 5, Seq. ID 6, Seq. ID 7, Seq. ID 8, Seq. ID 9, Seq. ID 10, Seq. ID 11, Seq. ID 12, Seq. ID 13, Seq. ID 14, Seq. ID 15 and Seq. ID 16.

40. A live mucosal antigen-delivery vector comprising a genetically engineered *Bordetella* expressing a heterologous antigen.

41. The live mucosal antigen-delivery vector of claim 40,wherein the heterologous antigen comprises a protective epitope.

42. The live mucosal antigen-delivery vector of claim 40 wherein the heterologous antigen is derived from an agent selected from the group consisting of *Leptospira canicola*, *L. grippotyphosa*, *L. hardjo*, *L. icterohaemorrhagiae*, *L. pomona*, *L. interrogans*, *L. bratislava*, canine distemper virus, canine adenovirus type 2, canine parainfluenza virus, canine parvovirus, rabies, herpes viruses, HIV, SIV, *Erysipelothrix rhusiopathiae*, *Pasteurella*, *P. multocida*, *Ascaris*, *Oesophagostomum*, pseudorabies virus, porcine parvovirus, pathogenic *E. coli*, including *E. coli* having K88, K99, 987P, and/or F41 adherence factors, *Clostridium spp.*, including *Cl. perfringens*, and *Cl. perfringens* type C beta toxoid, *Salmonella*, *Vibrio*, *Mycoplasma*, *Actinobacillus pleuropneumoniae*, *Haemophilus*, rotavirus, transmissible gastroenteritis virus, *Streptococcus sobrinus*, *S. mutans*, and *Bordetella* virulence factors.

43. The live mucosal antigen-delivery vector of claim 42 wherein the heterologous antigen is *Pasteurella multocida*.

44. The live mucosal antigen-delivery vector of claim 40 wherein the genetically engineered *Bordetella* further comprises a type III secretion system mutant.

45. The live mucosal antigen-delivery vector of claim 44 wherein the genetically engineered *Bordetella* is *Bordetella bronchiseptica*.

46. A vaccine comprising the live mucosal antigen-delivery vector of claim 40.

47. A method for vaccinating an animal susceptible to infection by wild type *Bordetella bronchiseptica* comprising administering an effective amount of a genetically engineered *Bordetella bronchiseptica* comprising a type III secretion system mutant.

47. A method for vaccinating an animal susceptible to infection by an agent comprising administering an effective amount of a *Bordetella* genetically engineered to express an antigen of the agent.

48. A method for cloning a DNA molecule having a sequence encoding a protein or polypeptide secreted by a *Bordetella* type III secretion system comprising isolating and characterizing a protein or polypeptide secreted into the medium by wild type *Bordetella* but not by a *Bordetella* type III secretion mutant.

49. The method of claim 48 wherein the method is selected from the group consisting of

a) 1)culturing a *Bordetella* type III secretion mutant which does not secrete type III secretion factors;

2) isolating the supernatant of the mutant culture;

3) precipitating and separating the proteins in the supernatant;

4) performing steps 1-3 with a culture of wild type Bordetella;

5) isolating polypeptides present in the wild type but absent from the mutant culture supernatant

6) determining the amino-terminal sequences of the isolated polypeptide;

7) isolating a DNA sequence comprising codons encoding the determined amino-terminal sequence; and

b) 1) immunizing a first species with a supernatant from wild type *Bordetella* culture;

2) immunizing a second species with a supernatant from a *Bordetella* type III secretion mutant which does not secrete type III secretion proteins or polypeptides;

3) expression screening filters comprising a *Bordetella* expression library by first blocking the filters with a solution comprising the serum from species B, then hybridizing the filters with a solution comprising the serum of species A;

4) incubating the filters with a solution comprising labeled antibody specific for species A antibodies

5) isolating a characterizing the clones obtained.

50. A DNA molecule encoding a protein or polypeptide secreted by a Bordetella type III secretion system obtained by the method of claim 48.

51. A DNA molecule encoding a protein or polypeptide secreted by a Bordetella type III secretion system obtained by the method of claim 49.

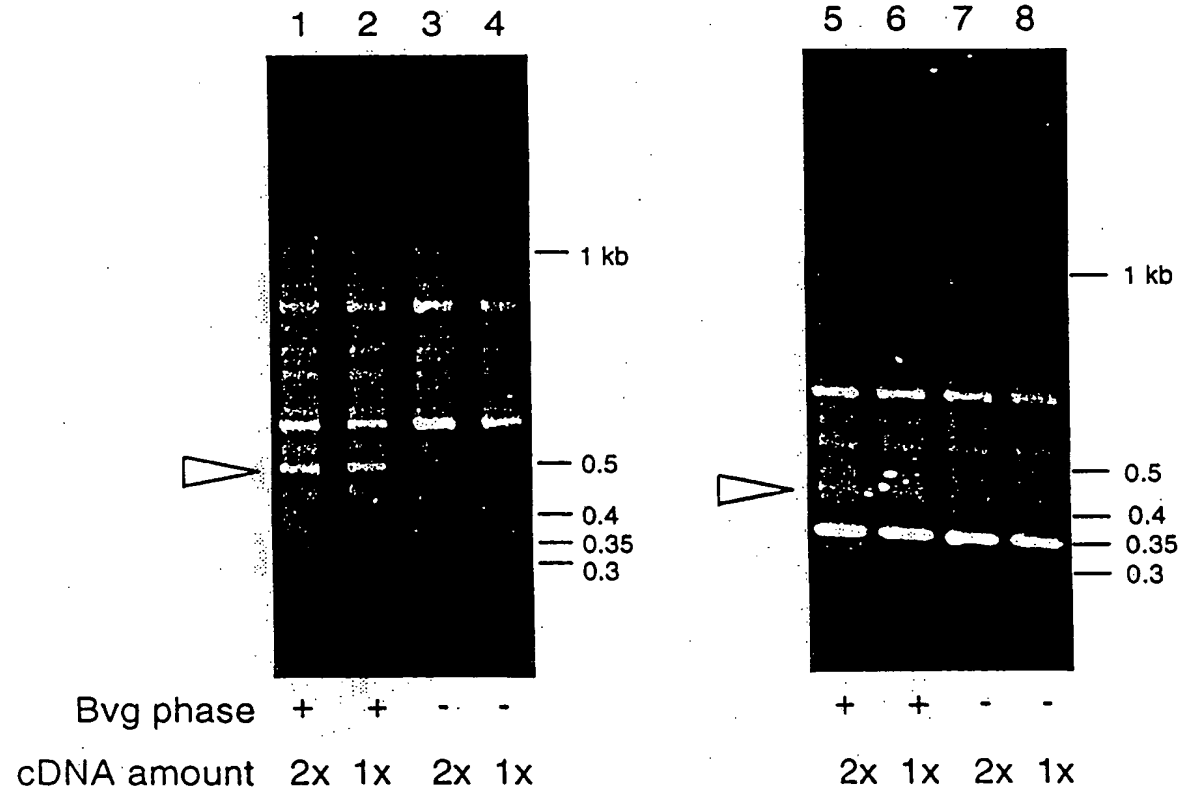
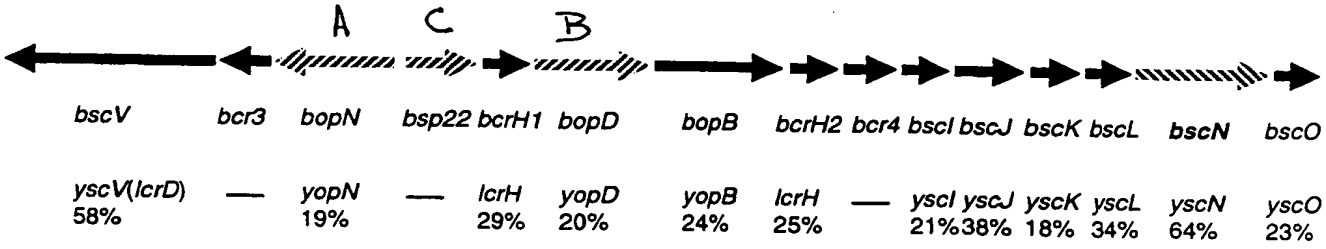


FIGURE 1

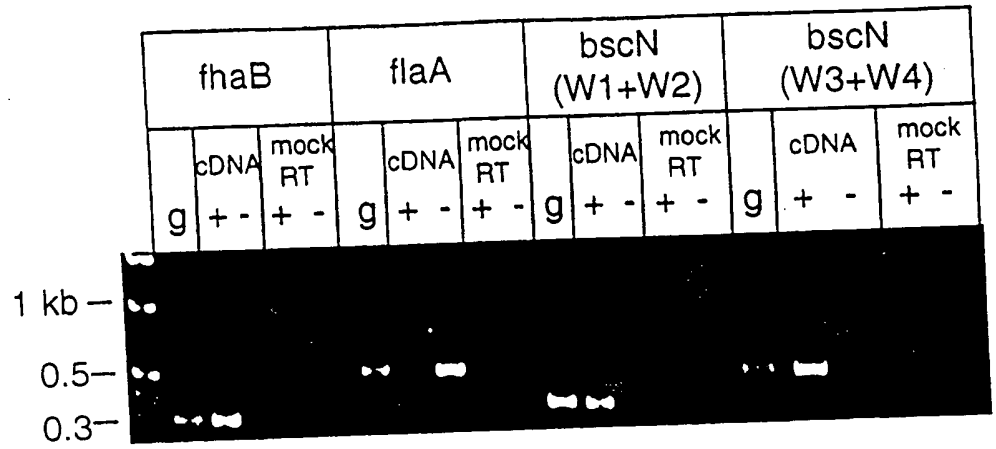
```
BscN  MRQYHYITEMMRVALQDLSTLRIKGRVVQVVGTIIKAVVPMVKIGEVCLLRNPGEDFEMH
      : .   : . .: ..     ..:.:::.::.:.:::::: :.::::.: :::: . . ..
YscN  MLSLDQIPHHIRHGIVGSRLIQIRGRVTQVTGTLLKAVVPGVRIGELCYLRNPDNSLSLQ

      GEVVGFVRDAALLTPIGDMYGISSATEVIPTGRTHMVPVGPGLLGRVLDGMGRPLDVAES
      .::.::    ::: :.:.:::::: ::: :::  :.: ::  :::.::::.:.:.:   .
      AEVIGFAQHQALLIPLGEMYGISSNTEVSPTGTMHQVGVGEHLLGQVLDGLGQPFD---G

                                                        Walker Box A
      GPL-HAHKFYPVFADAPDPLTRRIIHAPLELGVRVLDGLLTCGEGQRLGIFAAAGGGKST
      : : .   .::::. ::: :..:..: .:: ::.:.:::::::::::::::::::::::::
      GHLPEPAAWYPVYQDAPAPMSRKLITTPLSLGIRVIDGLLTCGEGQRMGIFAAAGGGKST

      LLGMLVKGAAVDVTVVALIGERGREVREFLEHELGPEGRRKSVIVCATSDKSSMERAKAA
      ::.:.:...: :::::.:::::::::::::::::.:.:: :: :: .: :::::. :::::::.
      LLASLIRSAEVDVTVLALIGERGPEVREFIESDLGEEGLRKAFLVVATSDRPSMERAKAG

                  Walker Box B
      YVATAIAEYFRDQGQRVLFRMDSVTRFARAQREIGLAAGDPPTRRGYPPSVFATLPKLME
      .:::.:::::::::.:.:.:.:::::::::::::::::::.:::::::::::::.::.:::
      FVATSIAEYFRDQGKRVLLLMDSVTRFARAQREIGLAAGEPPTRRGYPPSVFAALPRLME

      RAGMNQTGSITALYTVLVEGDDMNEPVADETRSILDGHIVLSRKLGAANHYPAVDVLASA
      ::::.. :::::::::::::::.:::::::::::::::::::.:::::.:::::::.::: ::
      RAGQSSKGSITALYTVLVEGDDMTEPVADETRSILDGHIILSRKLAAANHYPAIDVLRSA

      SRVMNAVVSPRHKYLAGRMRELMAKYQDVELLVKIGEYKQGADASTDEAIQKIGQINAFL
      ::::: .:: .:: ::..:.:.:::.:::::.::::..: :  .:.::::.: : ..:
      SRVMNQIVSKEHKTWAGHLRRLLAKYEEVELLLQIGEYQKGQDKEADQAIERMGAIRGWL

                                  444
      RQLTDEREAFEDTVLRMAEIIGPES
        : : :   :..: : . : .   .
      CQGTHELSHFNET-LNLLETL--TQ
                                  439
```

FIGURE 2A

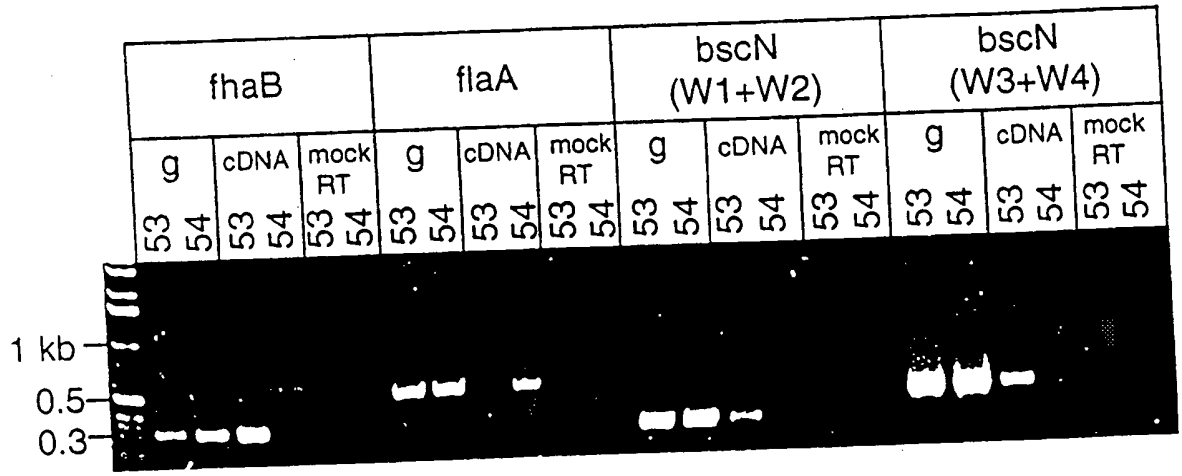
Fig. 3


A
C
B
bscV bcr3 bopN bsp22 bcrH1 bopD bopB bcrH2 bcr4 bscI bscJ bscK bscL bscN bscO
yscV(lcrD) 58%
yopN 19%
lcrH 29%
yopD 20%
yopB 24%
lcrH 25%
yscI 21%
yscJ 38%
yscK 18%
yscL 34%
yscN 64%
yscO 23%

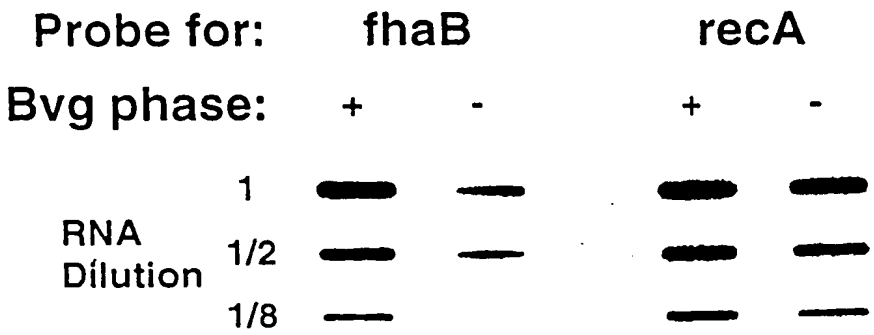
a



b



FIGURES 4A & 4B


Probe for:
fhaB
recA
Bvg phase:
+
-
+
-
1
1/2
1/8
RNA
Dilution


Probe for :
flaA
bscN
Bvg phase:
+
-
+
-
1
1/2
1/8
RNA
Dilution

FIGURE 4C

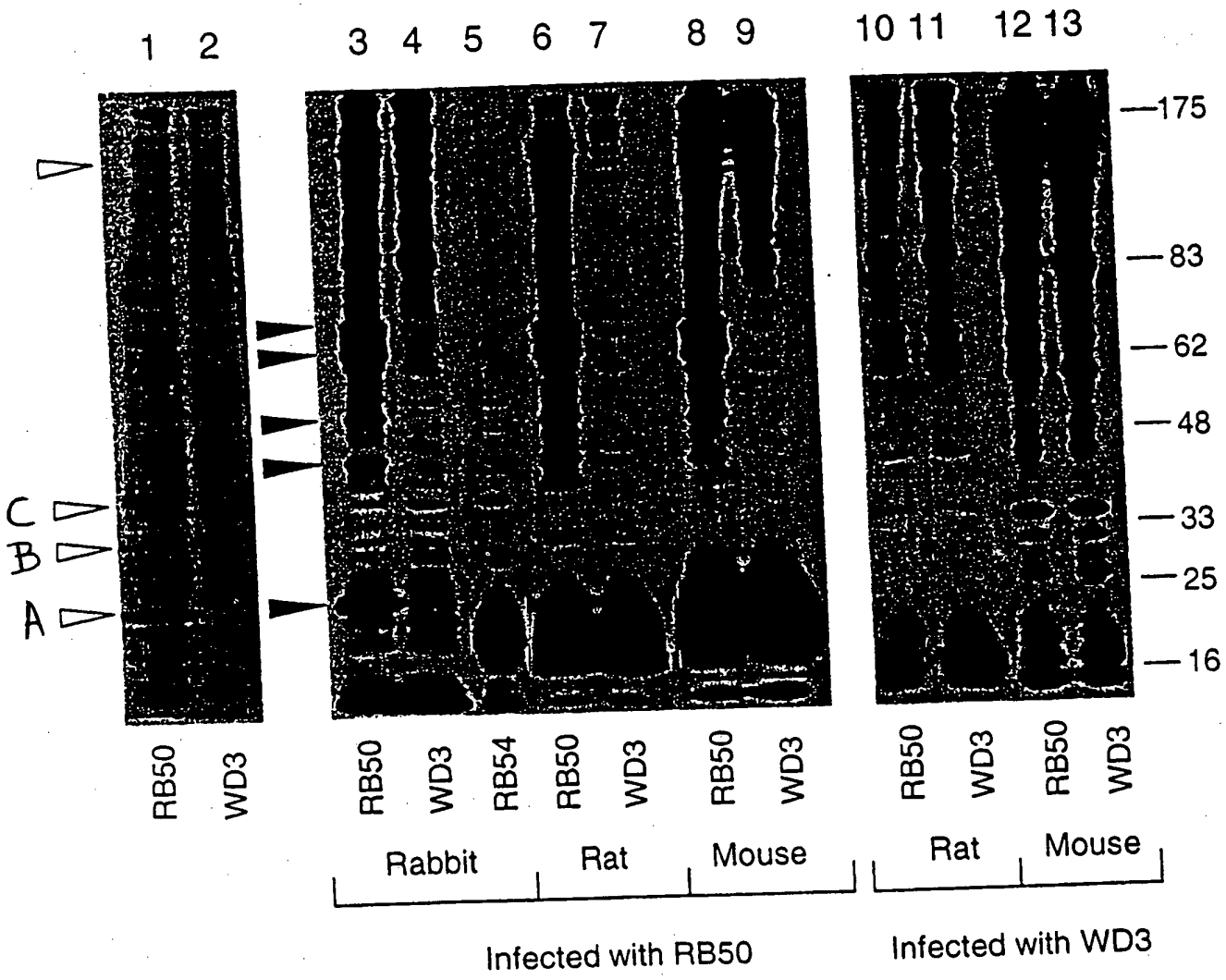
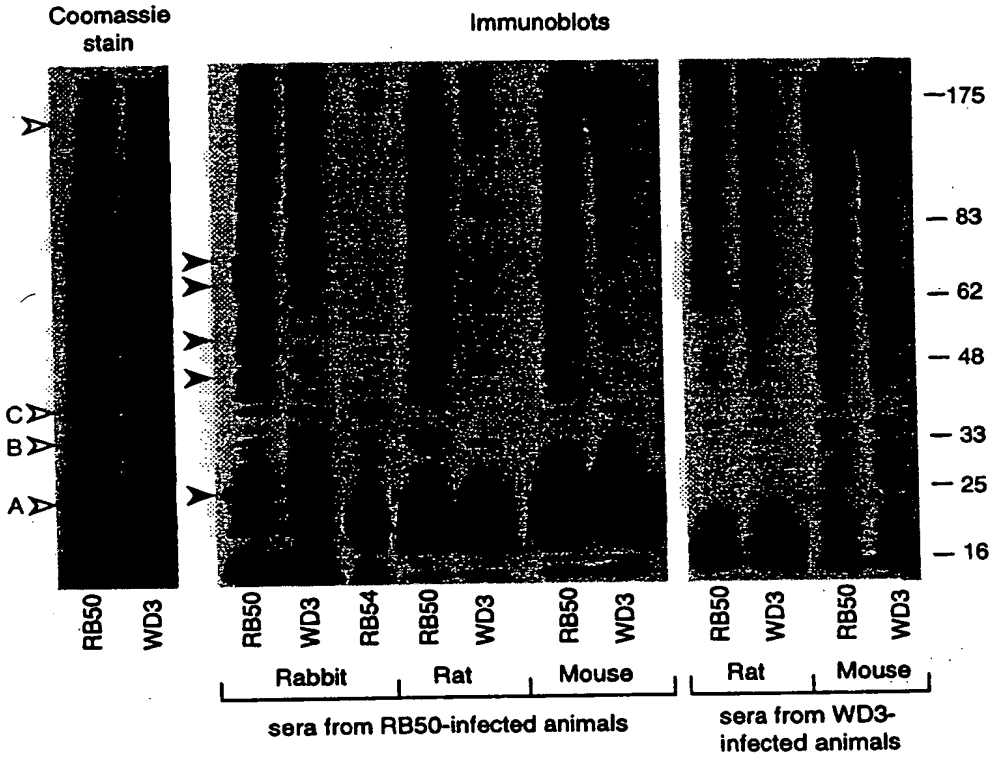

1 2
3 4 5 6 7 8 9
10 11 12 13
C
B
A
175
83
62
48
33
25
16
RB50
WD3
RB50
WD3
RB54
RB50
WD3
RB50
WD3
RB50
WD3
RB50
WD3
Rabbit
Rat
Mouse
Rat
Mouse
Infected with RB50
Infected with WD3
Total Stain
Immunoblots

FIGURE 5

Fig. 5'



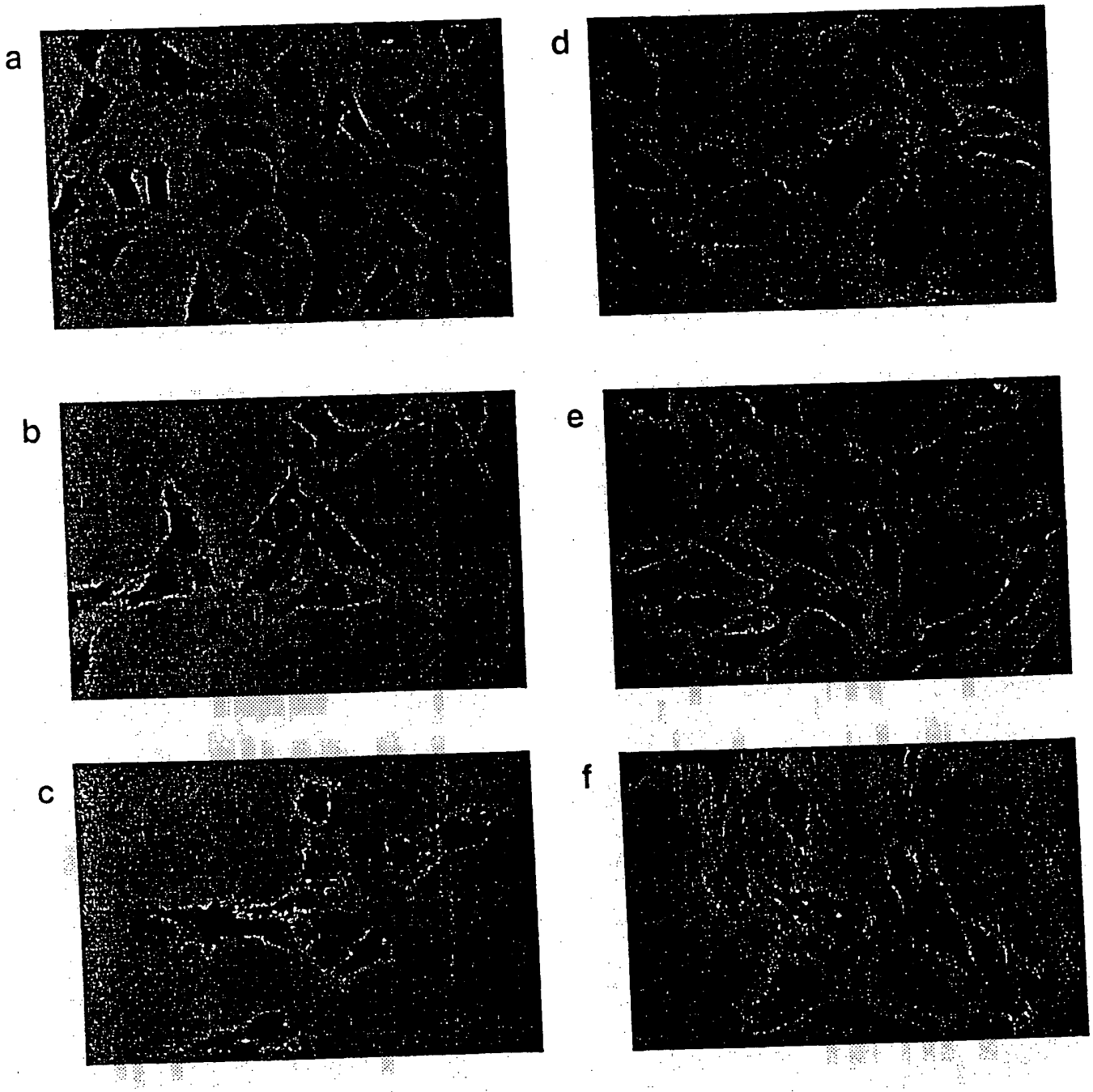


FIGURE 6

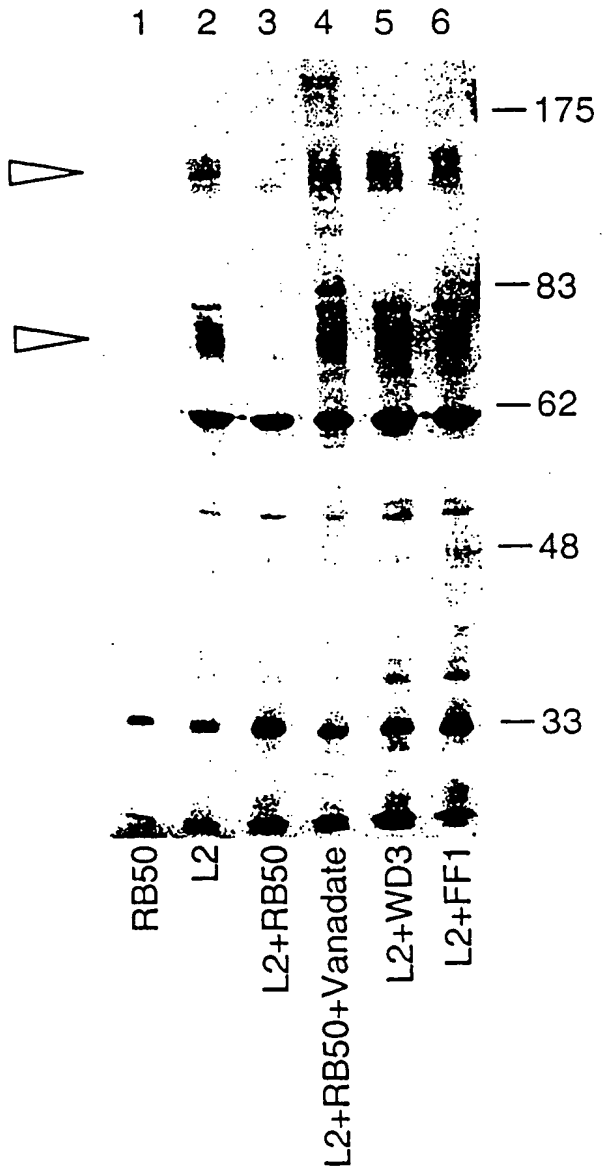


FIGURE 7

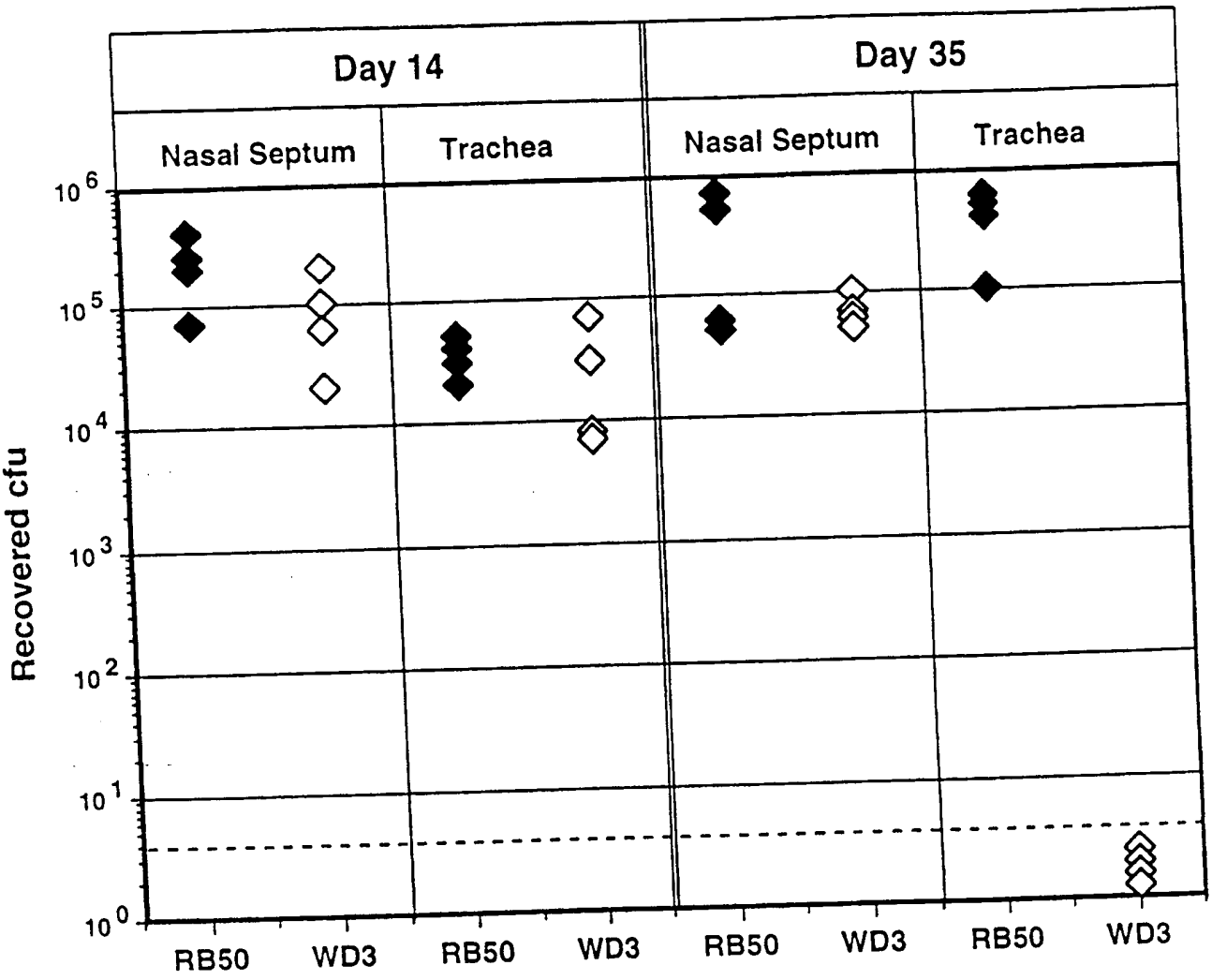

Day 14
Day 35
Nasal Septum
Trachea
Nasal Septum
Trachea
Recovered cfu
10^6
10^5
10^4
10^3
10^2
10^1
10^0
RB50
WD3
RB50
WD3
RB50
WD3
RB50
WD3

FIGURE 8

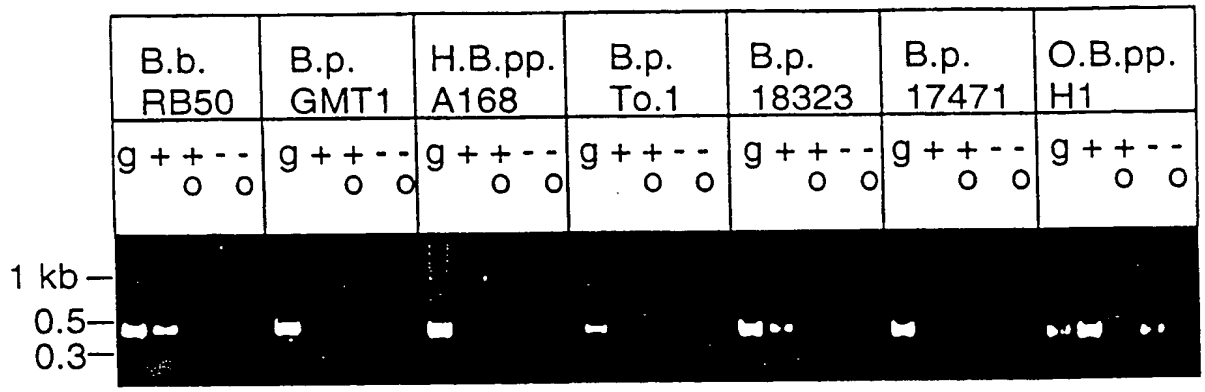
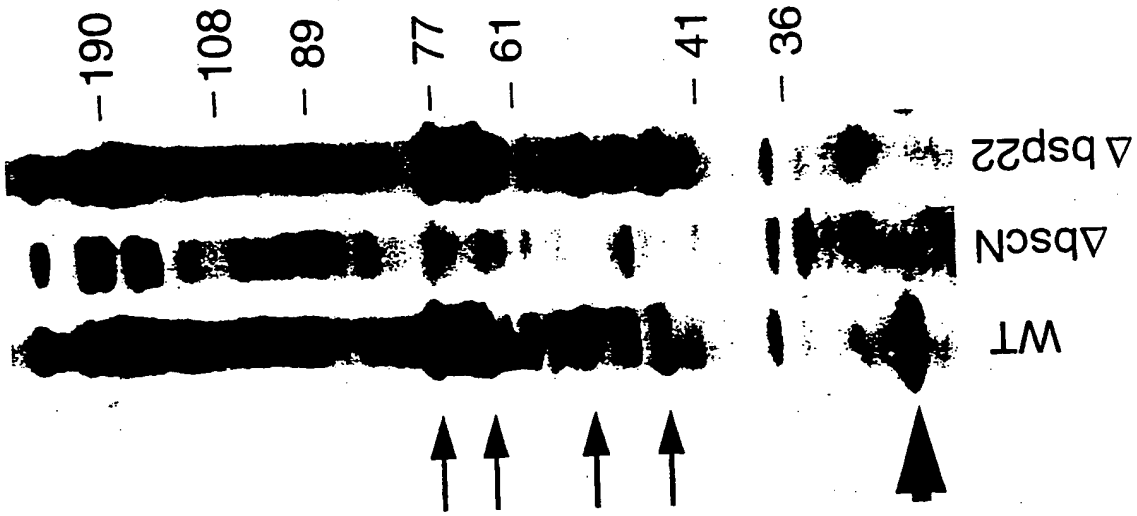
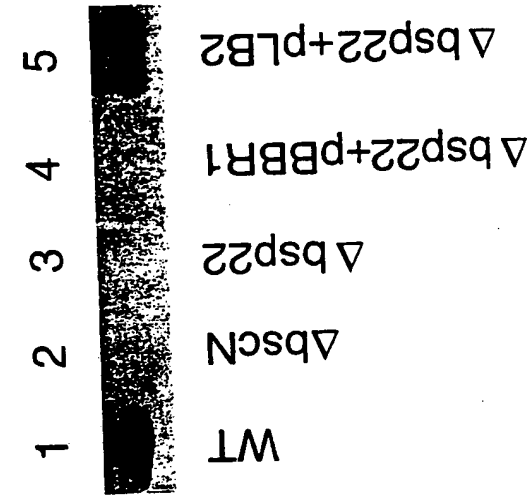

B.b. RB50
B.p. GMT1
H.B.pp. A168
B.p. To.1
B.p. 18323
B.p. 17471
O.B.pp. H1
g + + - - o o
1 kb
0.5
0.3

FIGURE 9

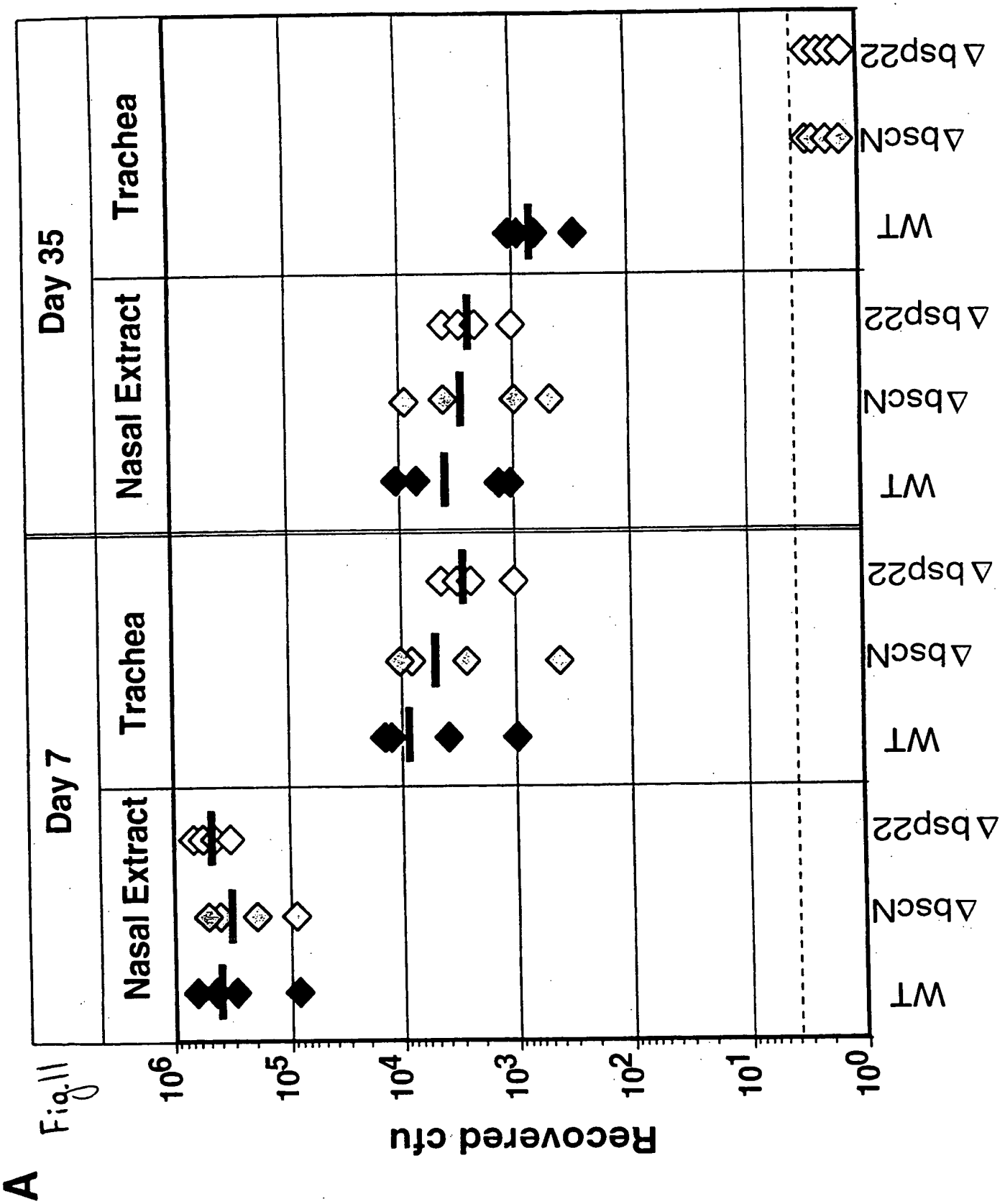

−190
−108
−89
−77
−61
−41
−36
Δbsp22
ΔbscN
WT

B


1
2
3
4
5
WT
ΔbscN
Δbsp22
Δbsp22+pBBR1
Δbsp22+pLB2

A

Fig. 10



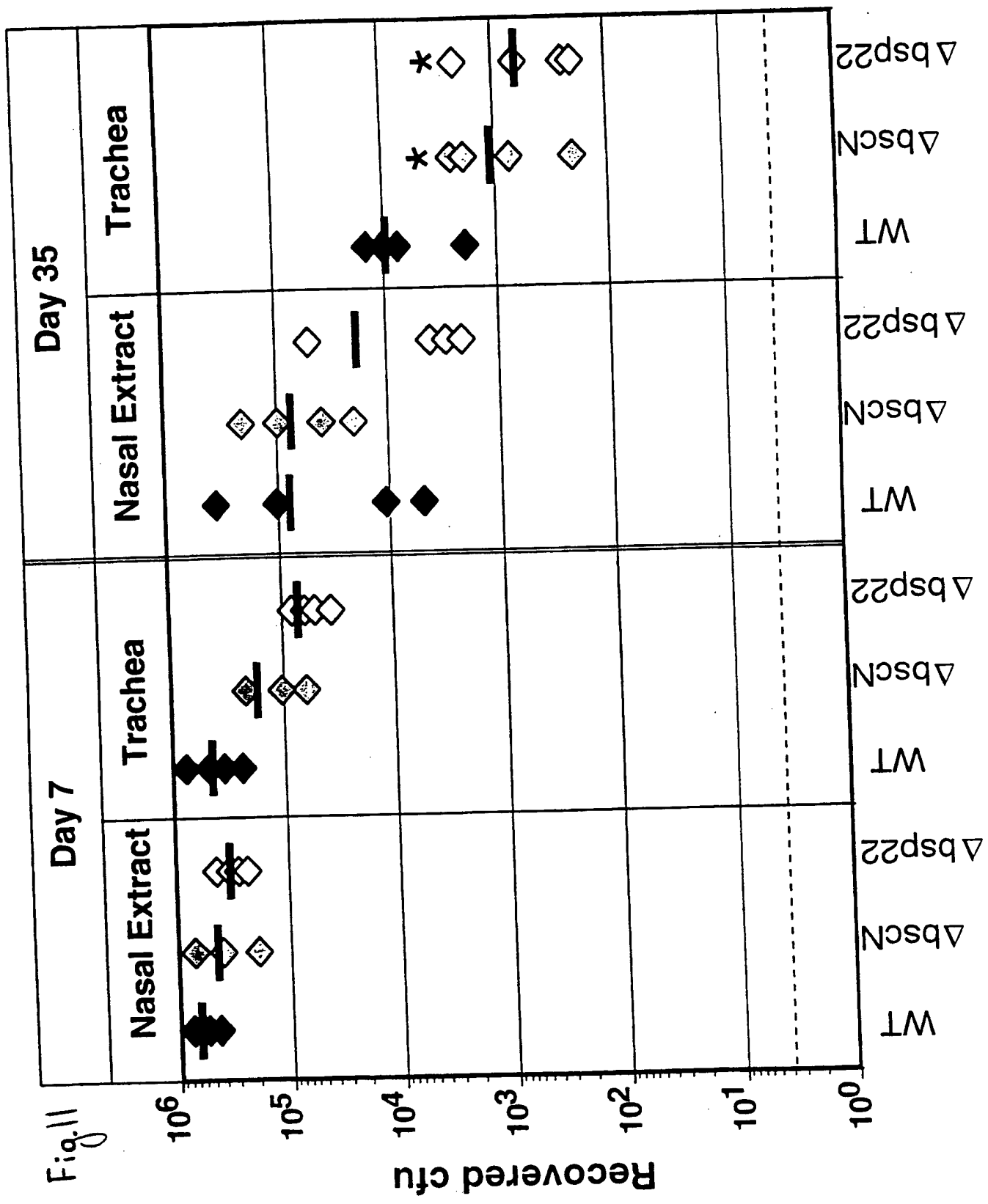


Fig. 11

B

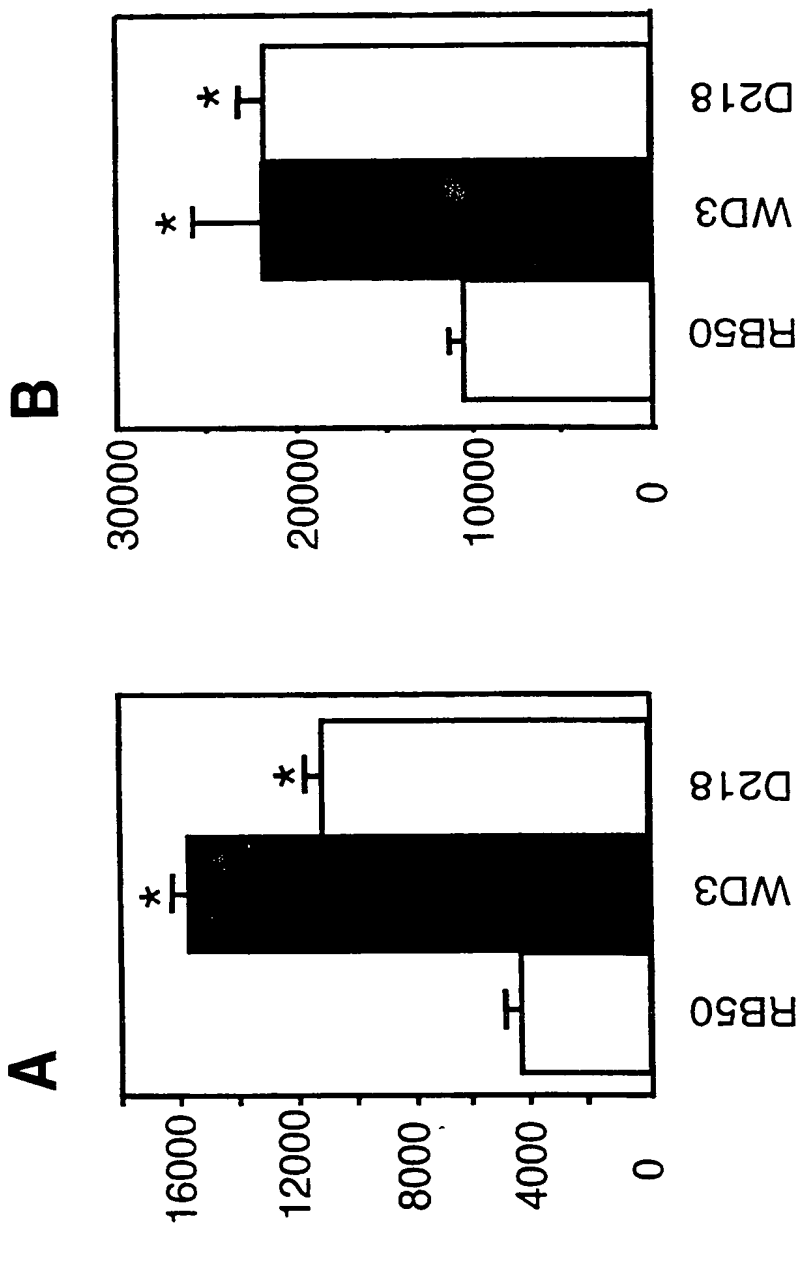
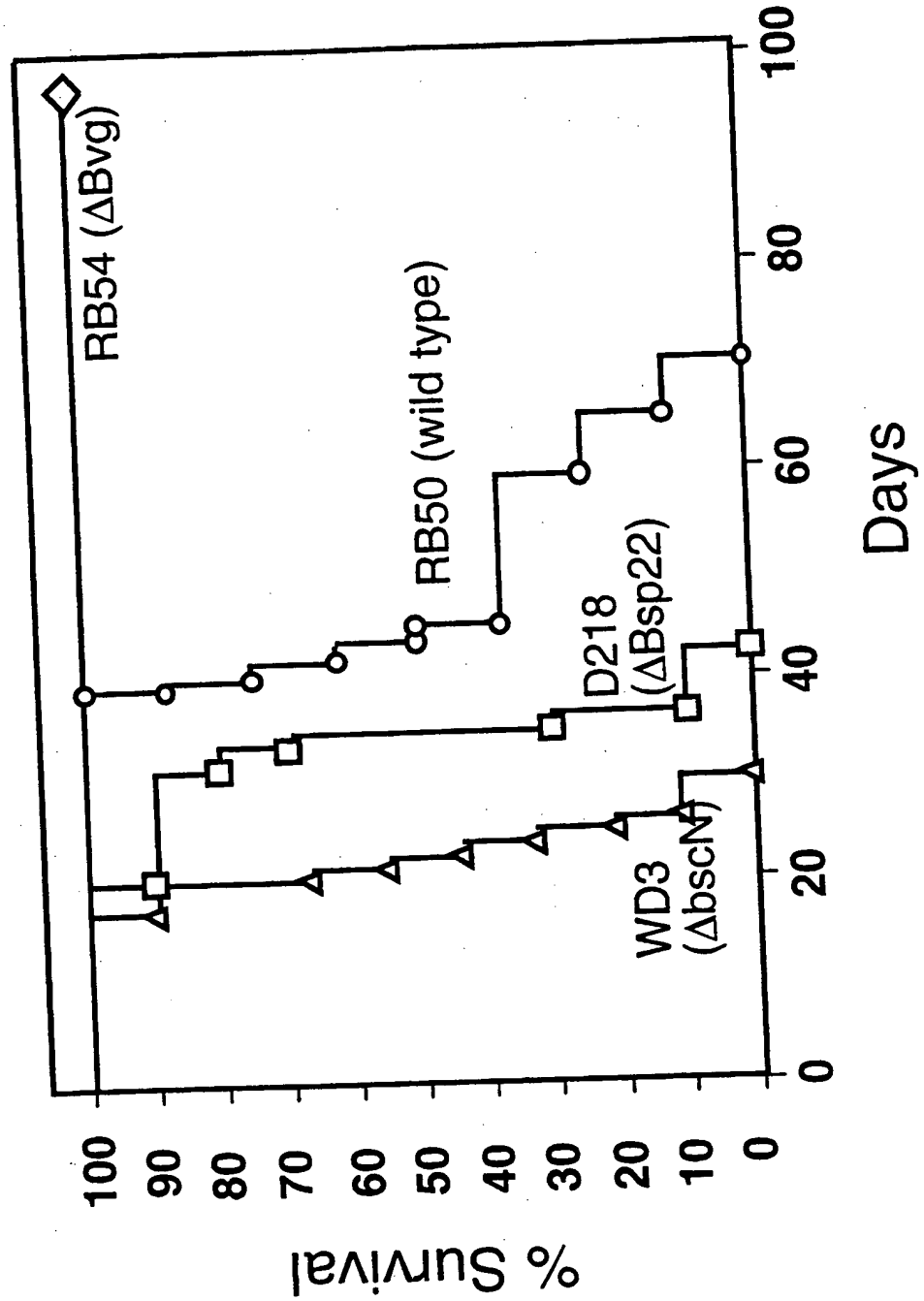


Fig. 12


RB54 (ΔBvg)
RB50 (wild type)
D218 (ΔBsp22)
WD3 (ΔbscN)
% Survival
Days
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100

A

Fig. 13

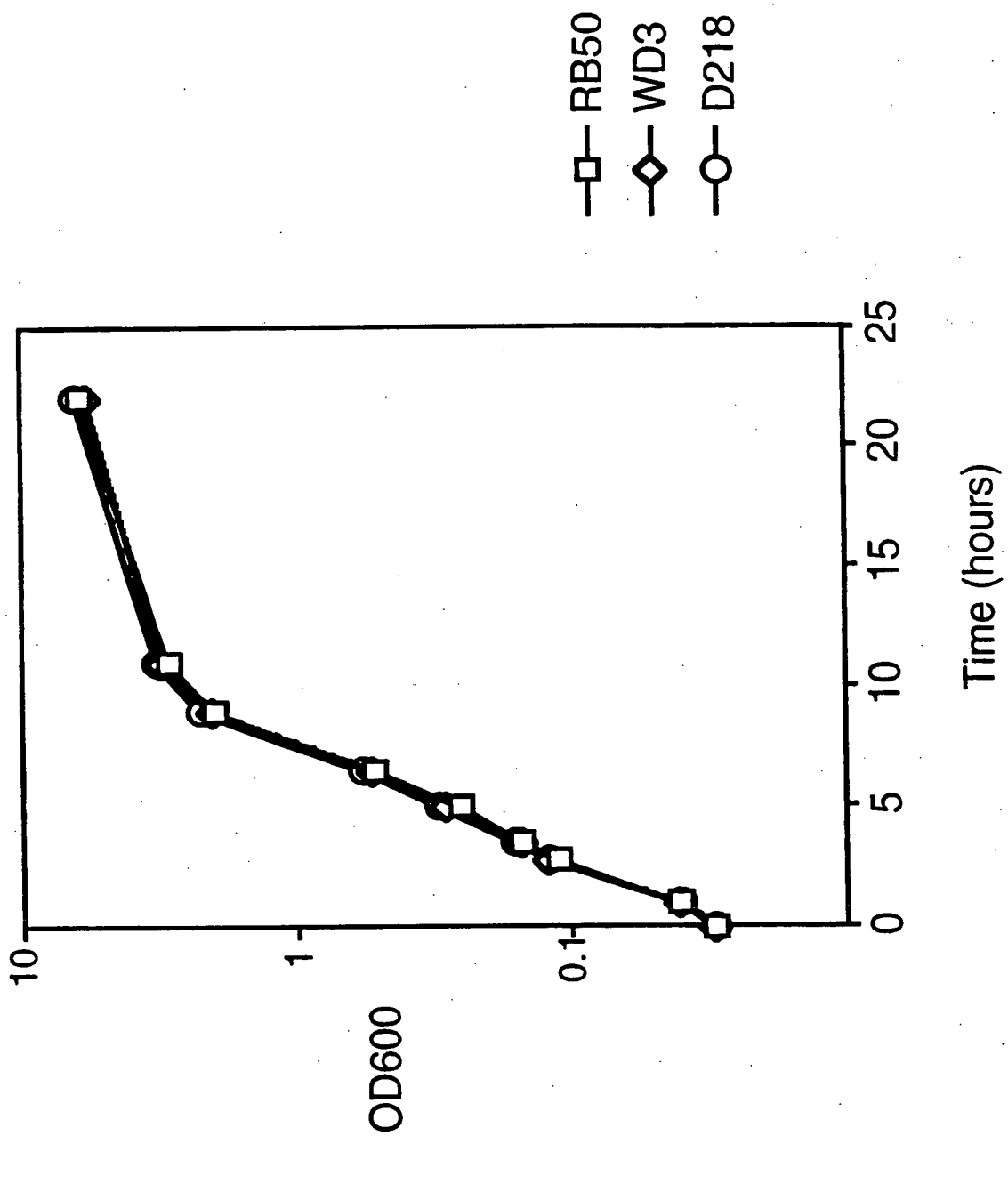


Fig. 13

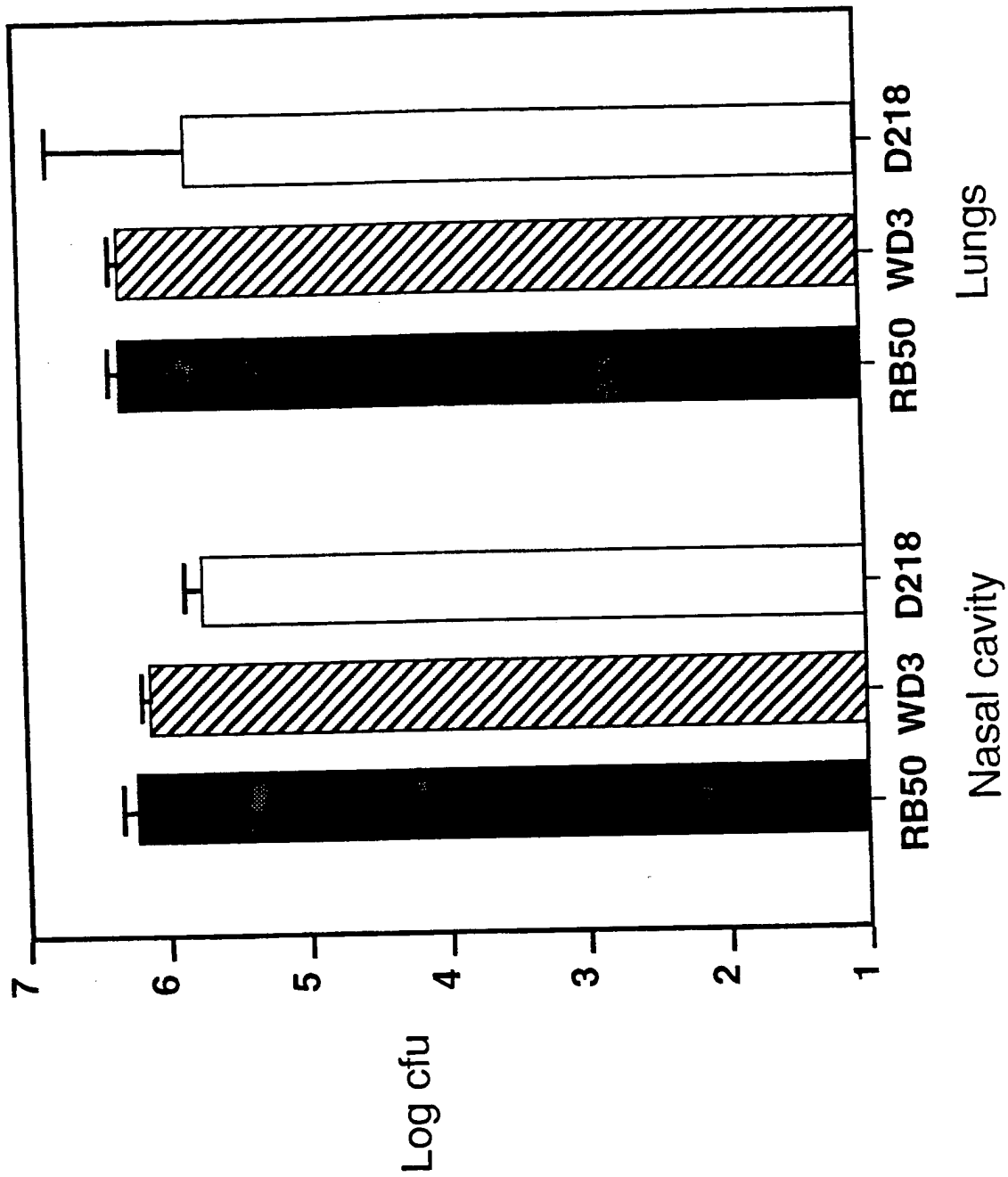


Fig. 13

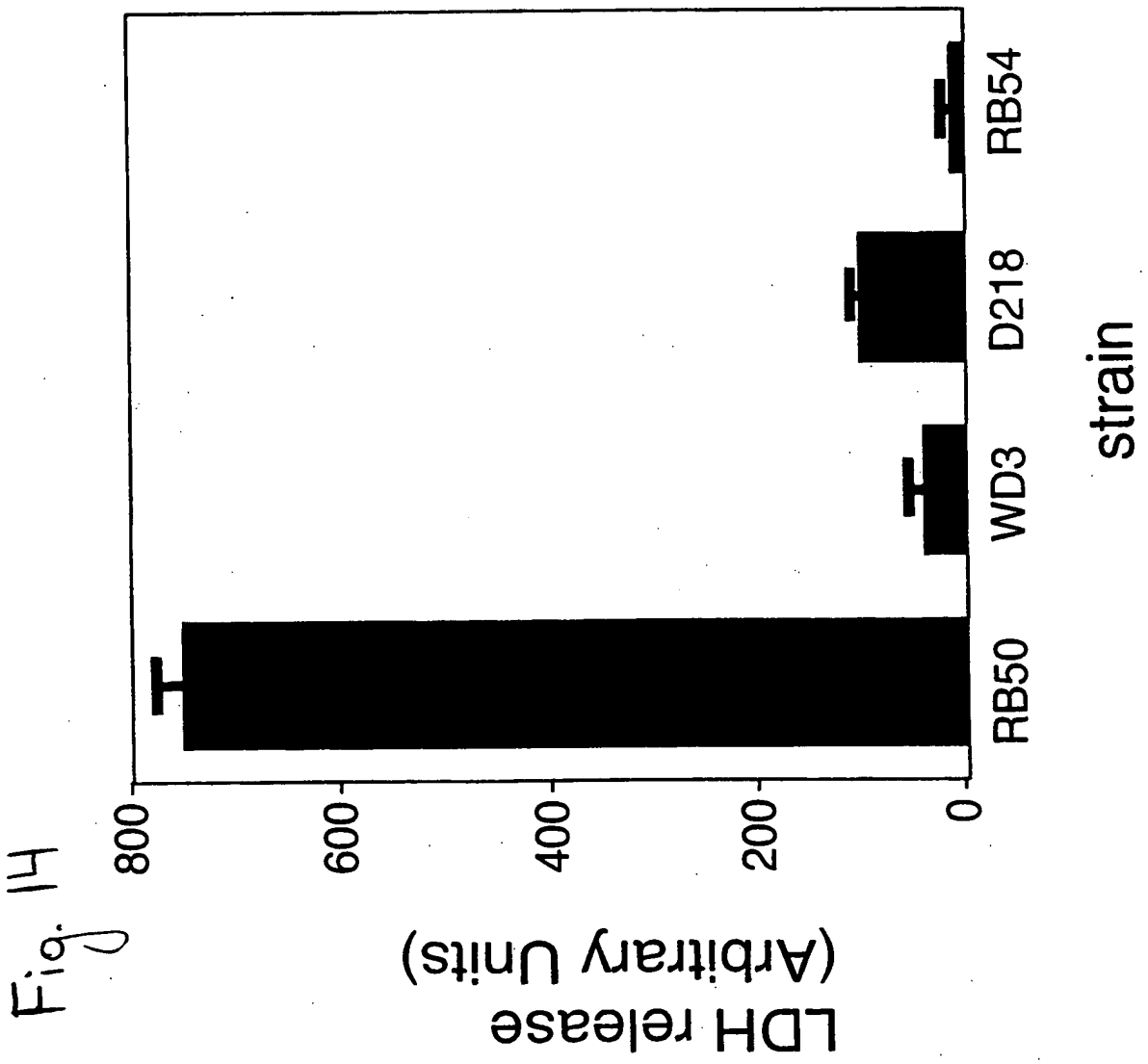


Fig 1H

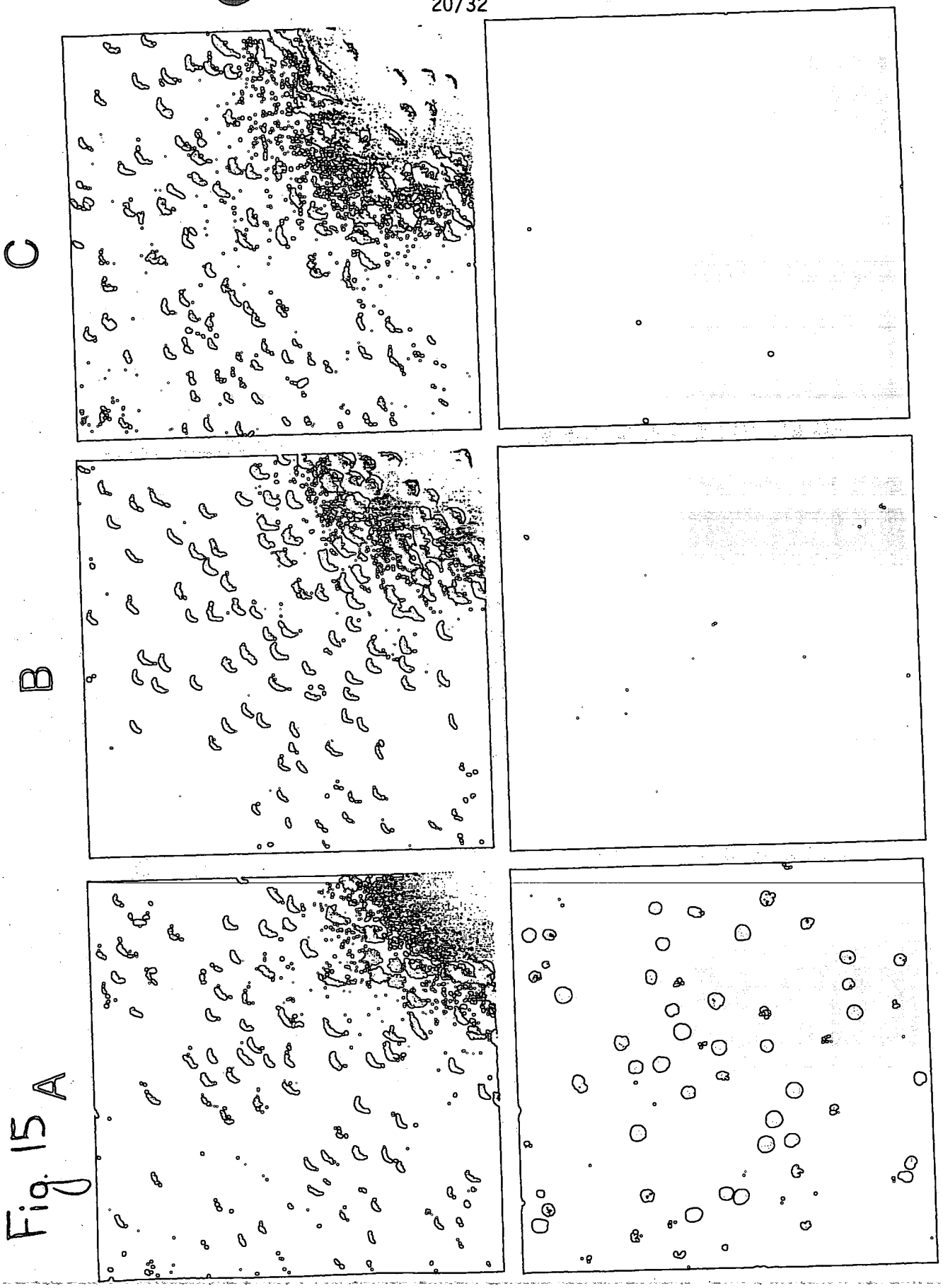
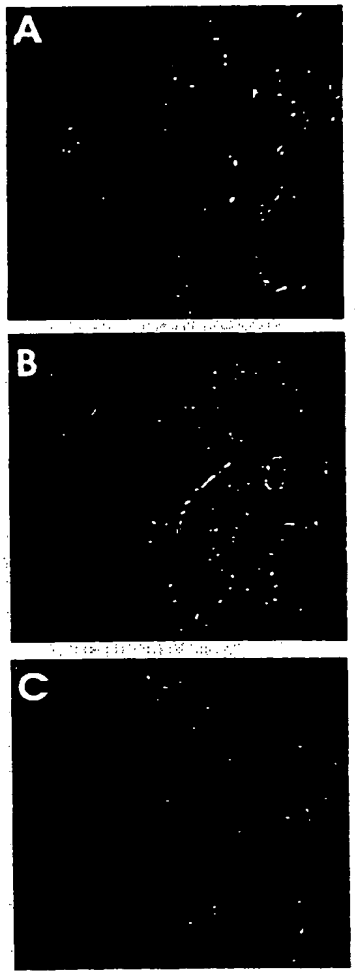


Fig 15

Fig. 16



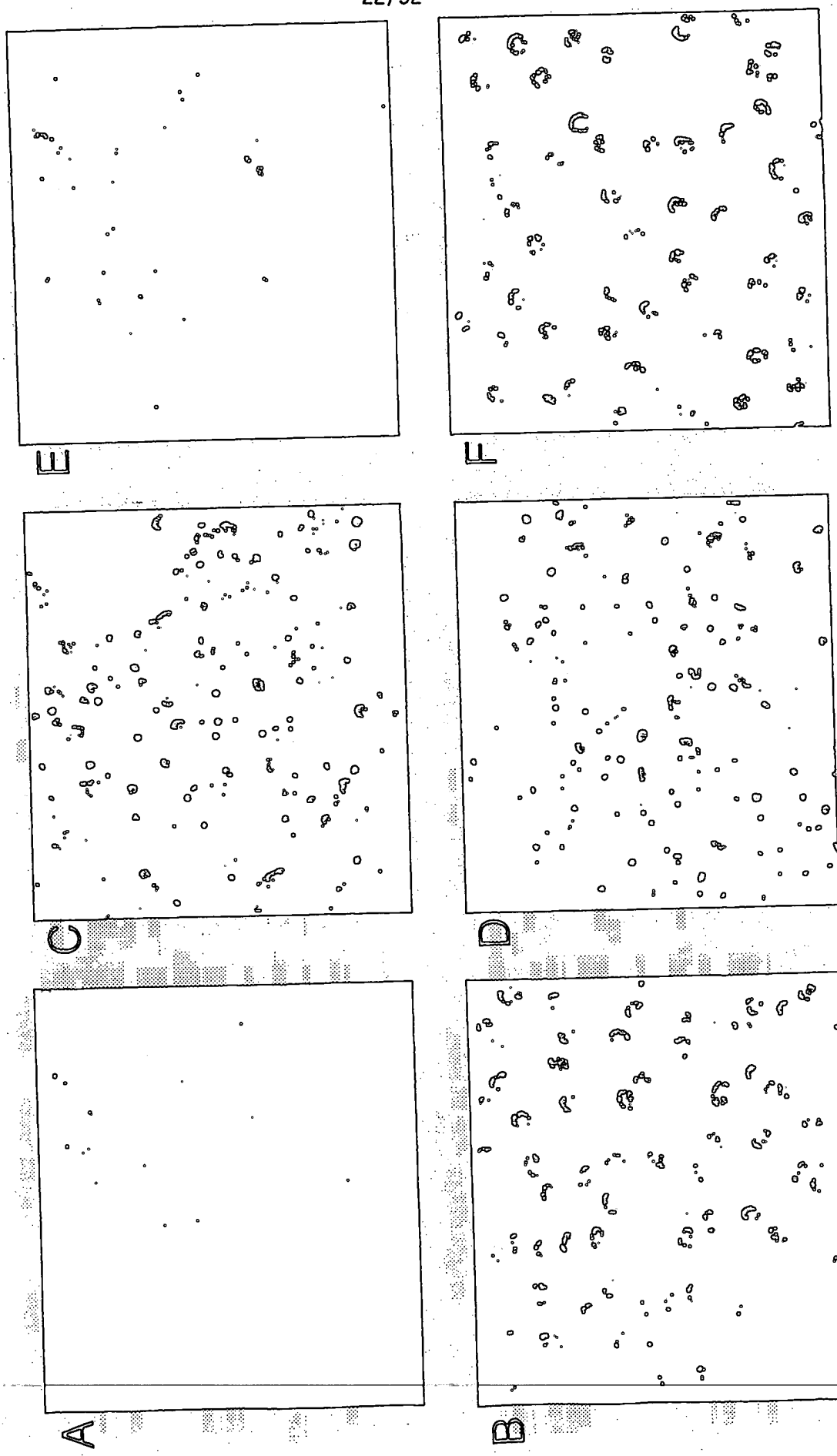


Fig 17

Fig. 18

GAATTCCAGCGCCGCCGTCATAGATCCACCCTGCCCAGGGGCTGTACATTGATCTCCGGCGTCAGTTCCTGGTAGGACAG
CACCGGCAGGGCGTAGAGATCGGCTTCGATCATCTTGCGCGTGTAGCGCCGGATGTCCATCGACGTCAGCAAGACGGGAC
GGCTCGCGCCGGCGGCCAGATCGCCGACACATTGGCGTATGTGCTCGACCAGCCGGCGTGTCGTGTCCGGATCGAGGGCG
AAATAACTGCCGGCGGCGGTCTGCCGGATGGCGGCGCGCACGGTTTCCTCGACCTTGGGGGCCAGCAGGTAGGCGGGCAG
GATATTGTGGCCGCTGGTGTACTTGTAGCTGATATAGCGCTTGAGTGCGATTCGGACATACTCCGTAAGCAGGACGGTAT
CCTTTTCCTTCTGGCCCCATTCGACCAGCGCTTCCAGGACGGCGCGCAGGTTGCGTATCGACACTTCCTCGGAAACAAGG
CGCTGCAGGATTTCGGCAATCTTCTGCACCGGCATGACGCGCAGGCACTCCTTGACCAGATCGGGAAATCGTTTTTCCAT
GGCCGAAAGCAGAAACCGGGTTTCCTGGATGCCGATGAAATCGGCTGAATATTTTTTCAGTACATATGCCAAGTGCCAAG
TCAGGATCTGGCTGATACCCAGGTAAGGAATACCTGCATCGCGCAAGGCGCCGGTCAGACTGGCTGCAACCCAGATCGTG
GGCGTATCGGGCAGAAAGGCCGCGCCCGTTTCGTATGCGATCCGCAGGGCCTGCAGGTTCTGCTCGGTGTCCCGCACCAG
CACGGCATCGTCGCGCAACATCCCCTGCGCCACCGGGATCTCCGACAGCACGATGGTGTAGGTATTGGCGGCCAGCGCTT
CGGTGAAGCGCAACTGGATGCCGGGAAATGGCACGCCCAAGTCGAAATAGAGCGCCCGCCGGATCTGCAGCAGATCGTCG
GTGAGGGTGGCCGGCTCGAACCGGGGCTGCAGCCGCGCGGCCACGTCGATGATCAGCGGGACGGTGGGAGCGAATTCCGC
CTGTCCATCCGCCGGCGCGCGGGTGCGGGGCAGGCCGTCGGCAGCCATGCCGGCGAGCGCGGGCCCGGCGCCTTCGGGCG
GACGCTGGGATGCGCGCAGCAGTACGAAACCGATGGTGCCCACCACGGCGGCCAGGGCGAAGAAGACCAGCGTGGGCATG
CCGGGAATGAGGCCCAGGCCTGCCGAGATCGCGCCGGCAATGACCAGGGCGCGAGGCTGCGCCAGCACTTGTGCGCCGAT
GTCGGTGCCTACGTTGGAGGGGCCATCCCCGGTCTGCACCCGCGTCACGATGATTCCCGCGCAGATGGCGATGAACAGCG
CCGGGATCTGCGCGATGAGCCCGTCGCCTATGGTCAGGATGGCATATGTCTGCACGGCCTCGCCGGCGCTCAGGCCGCGC
TGCAGCACGCCGACCAGCATGCCGCCAAGCAGGTTGACGGCAACGATGATCAGGCCGGCGATGGCATCGCCCTTGACGAA
CTTCATCGCGCCGTCCATGGCGCCATACAGTTGGCTTTCCTTCTCGACCGTACGGCGTCGGCGGCGGGCTTCGTCCATGT
CTATGGTGCCCGCGCGCAAGTCCGCGTCGATGGACATCTGCTTGCCGGGCATGGCGTCCAGCGAGAAGCGCGCGGCGACT
TCGGCCACCCGCTCCGCGCCTTTGGTGATGACCACGAACTGCACGATCGTGAGGATGAGGAAAACCACCAGGCCGACGAT
CAGGTTGCCGCCCACCACGAAGTTGCCGAAGGTCTCGATGATGTGGCCGGCATCGCCTTGCAGCAGGATCAGCCGCGTGG
TCGCGATGGAGATGCCCAGCCGGAACAGCGTGGTGACCAGCAGGACCGAAGGGAACGAGGAAAACGCCAGGGGCGAAGGC
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CAACGGCAGGATCATCATGAAGACGATCGCCACGATGAGCACGGCCAGTACGATGTCGTTGCGGCTGGTGGCCAGCGCCA
CCGCGCGTTGCAGGCGGAGAATGGATTTCTTGCTCGTCATGGCGGGGGCGCGCTCGCGCAAGACGCCGCCCGTAGCGCCA
TATAGTCACGCATGGCCTGGCGGGCGTCGTCGGGTCGGTTCAGCGCCTGCATGGCCTGCGCCCGGACCAGATGGCCGGCG
GCATCGGGTGTGGCGCGCAGCGCGCGCTTGTCCAGCGTGACCAAGGCCATGCGCGGTTCGTCCTGGTGCAGATAGCCCAG
CGCCAGGGCCAGCAGGGAGCGGCTGTCGATCGCGTCCAGGGCATCCAGGGCCGCCAGCAGGGCAACCGTCTTGCTCCATT
GGCGCTGCAACTGGTATTGGTGCGCAAGCAATTGCAGAAGTTCGCGTACCTGAAGGCTGGGGTAGGGTAGGGTATGCGGC
ATCATCCCTGGTAGAGCGCGCTGCGATACATGGCAGCCAGGTCACGCAGCTTTCCGGCCTCGTTGAGGACGTGCAAGGCG
CGGCTCAAGGCCGGCGCGGTGTTGCCGGCGTGCAGTTCCATGGCCCGGGACAGTTCGTCGCGCGCCCGCGCCAGGGCGCG
CTCGAACTGCGCGGGCTGGAGCAATGCGCCATCCGTCAGGCGGGGCCGCGCCGACTCGTCGAGCATCGCGCTCAGGTCCG
GACGCACGAGCAGGGCTTTCAGATGATCGACCGCGCCGGTGGCGGGCGGCTCGAGCCAGCGCTCGGGTGGCAGGGTGGGG
GCGGGCTCGCAGCGGGGGCCGGGAACGATGTGGTCGACGCCTCGCTCCAGGCCAAGGTGCATGGGATGCATGCCGGGTAC
GGCGCTGGACATGGCGTCACGCCTCCAGGCGGTCGAGCCAGTTCGTCAGGCCCAGCACCGCCAGGCGCAACGCCGCTGCG
TCGAGCGAGCGTTCGGGCAGTCGTGTCTGCGCGACGATCCAGTCCTCGCTGTCCTCCGACCAGAGTGACGTCTGGATGGA
TCGGGGCGCCGCGGCGCTGTCCATGGGCCCGTTTCCATGCCGCCAGCAAGACGGAGGCGGCGTCGCGCTCCGCCCGCTGG
GCCAGGTGGACCAGGGCCGCGCCGGCGACACATTCGACGCCCAGGCGGCGCCCGTTGGACAGCGCCAGCGACGCCGATCG
GGACGGCCCGAATGCCAGGCCCTCGATGCCGATATCCTGGCCGAACTGATGCAGCGCCTTATCGGCAGTATTCATGCGTT
CTCCATTGCTATCGCGTTGTCCAGCGCATCCTGCGCGGCCGCCAGGACGGTGGCGCGCACGTCCATGTCGGCGTAGATCT
GCGTGGGCAGGTCTTTGAGAATCTGGCGTACGCCGCCGAGGAATGCGATGCGCTCGGAGAGGGCATTCGCGCCGTGGCGC
TCTGCCAGCTTCTCGAAGCGCGCGGGCGCAATCCATTTGTCCTCGCTGATTCCCACAATATCGCGCATCAGGCCCTGGGC
GTCCGCACACTCCTGCGAGCCTGCGTTGCCCAGCCGTTGTTTCAGGGCATTGCATTCCTCCAGTACCGTGGCGGCCACCT
CGACTTGATAGAGATCGCTCGCCAACACTTGCAGCCTGACGCCGTCCGTCGACGGTGTCGCCGCGGCCAGGTCGTGTCCC
AGCGCCTGAATCAGCGCGCCCAGCGCGCCGTGGATGTCGTCGTTCCCATAGCGTTCCAGCACCAGGTCCAGCGTGCGCGC
CAACGACAGCTGGCCCAGGGCGATGTCGCGGTACGCGTGCTGGAAGCCGGCCAGCTCGTCAGCGGAACGCGCGAACGCGC
CGGCCGTGGGCAGGGTGTTGATGCCGGCGCGGATTTCGGGGCCATGGGCGAGCTCCAGGTCGGCCAATGCATCGCGCAGG
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ACCGGTAAACGCTTGCGCGGCCAGTTGCATGGGGGCGCCCCGGCCGCGCAGCAGCTCGGCGGTCAGGGCTTCCAGTTTTG
CCTGCGCGTCGGGGTCGTGGGTGTGGGAAAACAGTTCCGCAAGCTGCGCCGCGTCCAGCCAGAGCATCGGACGTTCGGCC

Fig. 18 (cont.)

GTGACCTTGCGTTCGGAGTGATGCTTTTCCTCGGCAGCCTGCGCCATGTGCAGGCTGAGCTCCTCGGCCGCGTCCGCCAG
CGATATGCCGGTGGGCGCCGGTGCGATGCGCTGGCCTTGCAGCCAGCCGGAGGAAGTGTTGGCCGAGGCGTCCTGGCGCC
CCTGCATGGCGGCGTGGAAGGGATTGGGGGCGGCATCGATACGAGTCATGGGGAGTCCTCGGAGAAGGAACCATTTGCCT
ACTGGTGCAGTGAGTGTCGCGCGCGCGGCCATGGTTCCCGGAAACGGGCGCGATATTGGGCAATTCGCAGCCTGGAACTT
GCCGCGGGCGCAGGGTTACTCAGCATGCGTCTTTCAACTCGAAGGAGCCCTCATGACCATTGATCTCGGAGTTTCACTCA
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CGCGCCGAACTCCTCACGGCTCAAATGCAGACCCAGGCCGAAGTGGTGCAGAAGGCCAATGAACGCATGGCGCAGCTCAA
CGAGGTCCTGTCCGCGCTGTCCCGGGCCAAGGCCGAGTTTCCGCCCAATCCGAAGCCGGGCGACACCATCCCGGGCTGGG
ACAACCAGAAGGTCAGCCGGATCGAGGTTCCTCTCAATGATGCGCTGCGCGCTGCCGGCCTGACGGGCATGTTCGAAGCG
CGCGATGGCCAAGTGACCGCCCCCGGCGGCCGGGGTACGCAGGTCGTGAACGGCACGGGCGTCATGGCCGGTTCCACGAC
CTATAAGGAACTCGAAAGTGCCTACACCACCGTAAAGGGGATGCTGGATACGGCGTCCAATACGCAACAGATGGACATGA
TCAGGCTGCAGGCCGCCAGCAACAAGCGCAACGAGGCTTTCGAGGTCATGACCAACACCGAGAAGCGGCGCAGCGACCTG
AACAGTTCCATCACCAACAACATGCGCTAAGCGCTGCACAAAGGGGTATTCCATGCAGGAGCAAGACATCCAATCCATCA
TGCGTGCCGCGGAAGAGCTGGTCGAGCAGACCCGCAAGGCGTTGTGCAGCGTCGACGAGATCTACGCCCACGTTGGCGTC
GACCCCGCCCGCCTGCGCAACCTGGCGGTCGAGCAGGCAAGGATAGAGGCCGAGGCCCAGGCGGCGTTCCGTGATGACCT
CGCGGACATCGAGCGCGAGGCGGCGCGCGTCAAGGCGGCCAGCACCGATGCGCCGCAGGCCCGCAGGGTGCTTCACAACC
ACGTCTGAGCGCGGAGGCCTTCCATGCCAAAGTCAGCCGAGCAGGGCGGCTCCCCGGCGTCAGCTTCGCATGAGGCGTTG
CGCCATATTCTTGACGCAGGCGCTTCGATGGGCAGCTTGCAGGGGTTGGACGAGGTGCAACAGCAGGCGTTGTACGCGAT
CGCTCATGGCGCCTACGAACAGGGCCGCTATGCCGACGCGTTGAAAATGTTCTGCCTGCTGGTCGCGTGCGATCCGCTGG
AAGCCCGTTATCTGCTGGCCCTGGGCGCCGCGGCCCAGGAGCTGGGGCTGTACGAGCATGCCTTGCAGCAATACGCGGCC
GCGGCGGCTTTGCAAGTTGGATTCCCCCCAGGCCCCTGTTGCATGGCGCCGAGTGCCTGTATGCGTTGGTTCGTCGCCGC
GACGCCCTGGATACGTTCGACATGGTGCTTGAGTTGTGCGGGTCGCCGGAGCATGCGGCCCTGCGCGAACGGGCCGAGTC
GCTGCGCAGGAGCTATGCACGTGCCGACTGAAACGGCGCCATGTCCGCCGTCAAGATTTCAATTCGAGGAGGTTAGATAT
GTCTGTTTCTCCGACTTCGCCCGGCTCTTTCGGGGCCGGCCCTGTCTTTGACTCCGAATTGCAGGCCCCGGCCCCGTCGG
CGCAGCGTCGCGGCGGTGCGGCGCCTGTGCCGCCGCCCGTCGATCGGCGCGGAGTCGAGCCGGGAGATCCCACGCTGGGC
ATGCTGCCCGCGCCCGATTTGCTCGCGGGGGGCGCCGTCAGCCGCACCCGCGCGGCGCTCGACGATCTGGACGCAGCACG
GCTCGGTGAAGACATCTACACCTTGATGGCGGTGTTGCAACAGGCCAGTCAACAGATGCGGGAGGCCGCCCGTATCGCTC
GTGATGCCGAGGCCACGCGGCAAACGCAGGCTATCGGCGATGCGGCCAGCCAGATGCGCCAGGCGGCCAACGAGCGCATG
GCCGGAGCGATCGTGGCGGGCGCCATGCAGATAGCGGGTGGTTTCGTGCAGCTGGGGGCGGGCCTGGCAGCGGGTTTGCA
GGCCATGGGTGGCGCAGCTGCGCAAGCCAAGGGCGCCGCATTTTCCGAGCAGGCCTCGACAAGCCGCAAGGTGGCGGCCG
GCTTGCACGATGCCCCCGAGCTGCAGGCAACGGTGCAGGCCCGCGCAACCCAGCTCGAAGCGCAAGCGGCTTCGTTTGGT
GCTGACGCGGCTCGTTCGTCGGCAAAGTCGCAGCGCGTATCGAGCGTTGCCCAGGCCGGCGCCGCGACGGCCGGCGGTAT
CGGCGGCCTGACCAGCGCCGCCCAGGAACGCCGCGCCGCCGAGCACGAGGCCAGGCGCGCGGAGCTGGACGTCGAAGCGA
AGGTGCATGAAACGGCCTCGCGGCGGGCCGACGAAGCCATGCAGCAGATGCTCGACATCATCCGCGGCATCAGGGAAAAG
CTGGCCGGGATGGAGCAGTCCCGCAGCGAGACCGCCCGTAGCGTGGCCCGCAATATCTGAGTGTCCGGCTCCAACCTTCA
ATCTTGAGGATGACCGTCATGAGTACGACCATATCCACAGCCCCGAGCGGCGCCGCGCTTGCGCCGTCTCGCATAGATAT
GCGGGCACCGGAGCCCGGGAGTGCCGGCGAAGGCGCCGGCATCCTGGCGCCGGTGACGACGCTGGCTCTGGCGGCGGGCC
GGCCGGCTTTTCCAGCGTCACCGTCGCTGCGCACCGCGCCCGTCCTGGATCCGCCAGTGCGCGATCTCAGCCCCGCCGAC
TTGGCCGACCTGCTGCGCGTCTTGCGATCCAGGGCGGTGGACGGGCAGTTGGCCACGGCGCGCGAGAACCTGCAGGACGC
GCAAGTCAAGGCGAAGCAGAACACCCAGGCCCAGCTCGACAAGCTGGACGCATGGTTTCGGAAGGCCGAAGAGGCCGAGA
GCAAGGGATGGCTGAGCAAGGTGTTCGGCTGGATCGGCAAGGTGCTGGCGGTCGTGGCATCGGCCCTGGCGGTGGGCTTT
GCCGCCGTCGCCAGCGTGGCCACCGGCGCGGCGGCCACACCCATGCTGCTGCTCAGCGGCATGGCACTGGTCAGCGCCGT
GACATCGCTGGCCGACCAGATATCGCAAGAAGCGGGAGGCCCGCCTATCAGCCTGGGCGGGTTTCTCTCCGGGCTGGCCG
GACGTCTGCTGACAGCGTTGGGGGTGGATCAGTCGCAGGCCGACCAAATTGCCAAGATCGTCGCCGGCCTGGCCGTGCCC
GTCGTCTTGCTGATCGAACCCCAGATGCTGGGCGAAATGGCGCAAGGCGTGGCCAGGCTGGCTGGCGCCAGCGATGCCAC
CGCGGGGTACATAGCCATGGCGATGTCCATCGTGGCGGCGATCGCGGTCGCCGCGATCAATGCCGCCGGTACAGCCGGCG
CGGGTAGCGCTTCGGCGATCAAGGGGGCCTGGGATCGGGCCGCCGCGGTAGCCACCCAGGTCCTTCAAGGGGGTACGGCA
GTGGCGCAAGGCGGCGTCGGCGTGTCGATGGCAGTCGATCGCAAACAGGCCGATTTCTTGCTCGCCGACAAGGCGGATCT
GGCGGCGAGCCTGACAAAACTGCGGGCGGCCATGGAGCGTGAGGCGGACGATATCAAGAAGATCCTGGCTCAATTCGACG
AGGCCTATCACATGATCGCGAAGATGATCAGCGATATGGCGAGTACGCACAGCCAGGTCAGCGCCAACCTCGGGCGGCGC
CAGGCGGTGTAGCGCCGGGCGCTCAAGGAATTTTCATGACTGTTCACGACGACGCGGCCGCGGCGCTGCGCGCCCGGCTG
GATGCGCTGCCGGGCAGCCGGCGCCTGACGGCCGAGCAATTGGAAGTGATTTACGCGATGGCGTATGCGCACGTCGCCAG
GTGCGAGTACGGCAAGGCGCTGCCCATCTTCGCCTTCCTCGCGCAGTATGGTCCCACGCGCAAGCATTACTGGGCCGGGC
TGGCGCTATGCCTGCAGAAGACCGACCGTCCCGACGAGGCGCGCAATATCTATGCGTTGATCCTCACGTTCTATCCCGAT

Fig 18 (cont) 2

TCCGCGGACGCCGTGTTGCGCACGGCCGAATGCGAGCTGGCGTTGGGTGAGAACGAACGGGCACAGGCGGCCCTGTTCGG
CGCAATTGCCATCGATGCAGAAAGTGGGCAGCCAGGTCCGGTCTCGCACCGTGCGCGCGCTTTGCTCGATCTTATTTCAG
TTTCACATCCGGAGTAACTCCATGCATTCAGACTCAGGTTCAGATTCAGGCTCAGACTCAGGCTCAGGCTCACCCATGGC
CTCGTCGATACATCCATCGGAACCGATACAGCCGATGGAGCATGTGCTCGAGGAGGCCGACGCCCGCCTGCTTACCGAAG
TGGGCTTTCTGGCGGCGGCCGTCAGCGATCTGACGCGCGCGGACGCCATTTTCAATGCATTGCAACGTGTACGGCCGGGC
CGGACGTATCCCTGCATCGGCCTGGCGGTCGCCCGCATGAACGCCGGGCTGCCCGACGAAGCCGCCGAGATCCTGGCGAA
TTTCCAGCCGGCACAGGCGGAGGACCGCTCGGAACTGGACGCCTGGTGCGGGTTCGCCCTGTTGCTGGCCGGCCGCTCGG
ACGAGGCGCGCCGCATGCTGCAGCGAGCCATCGATGCGGGTGGCGAGGCGGCAAGGCTGGCGCAGGTCGTGTTGGACAGC
GGACCCGCCATGATGCGGCCCGCGCCGTTGCAGTCCGAGCCATTACCTGGAGCTCCTGGATGAATTTGGATCTGACGGCG
ATCAACGCCGTGCAGGAACGGCTGCTCGCTCGATCGTTCGACATGCCGCGGTCTCCCGCGATGGCGGATCAGGCGCGCTT
TGAGTTGGCGCTGGGCGAGATGCCCGGCGCATCGGCCCCGAACGGGGCGATCGCCCCGGCACCGGCCGAAGGCCCGGCGG
CGCCGGTCGCGCTCGGCGAGCCGCTGGGCCGCCGCATTCTTGGACAGTTGCGCGGCGGCCTGGCCGATGTGGCAGGAAAA
TGGCGGGCGGTGCAGACGGGCTTGGCCGAGGTGAGCCAGGCGCCTACCGTGGTGGGTATGCTCGATCTGCAGGCCAGGTT
GCTACAGGCATCCGTGGAGTACGAGTTGGTGGGCAAGGCAATAGGGCGCGCCACCCATAACGTCTACACGCTGGCGAGAA
TGTCATGAACGCCATCGGGGCGATCCAACGGTATCGGCGCGGCGCGGGATGGGCGGCCCTGGCGCTCGCCCTGGCGCTGC
TGGCCGGCTGCGGCGCCCGCGTCGAGCTGTTGGGCGCGGCGCCCGAGAACGAAGCCAACGAAGTATTGGCGGCGCTGCTC
GAGGCAGGCATCGCTGCGCAGAAGCAGTCCGGCAAGGCCGGCTACGCGGTTTCGGTGCCGGCCGAGGCGGTGGCCCGGTC
GCTGGAGATCCTGCGCGCAAGCGGCCTGCCCCGCGAGCAGTTCGACGGAATGGGGCGCATATTCCGCAAGGAAGGCCTGG
TTTCATCGCCGCTCGAAGAGCGCGCCCGCTACATTTATGCGCTATCTCAGGAATTGGCCGACACCCTGTCGCAGATCGAC
GGCGTGCTCAGCGCCCGCGTGCACGTGGTGCTTCCCGAGCGCGGCGCGGTCGGCGAGCCGGCCACCCCTTCGACGGCAGG
GGTGTTTCTCAAGTACCGCGACGGACAGAGCCTCGACGCGCTCGTGCCCGAGATCCGCAAGCTGGTCACGCATGCCATCC
CGGGCCTGGCCGAGGACCGTGTATCGGTTGCCCTGGTGGTGGCCCAGCCCGTTCAGGCCGCACCCGTGCCGGTCGCGTGG
CGCCGCGTGCTTGGCGTACAAGTCGCGGACGGATCGGTCCTGAGATTTTCGCTGTTGCTGCTGTTGTTGCCGGTGCTGTG
CCTGATAGTGGCGGGGGCCACGCTCTACGCCTGGCGCACGCGCTGGTCCCGCGGCGAAAGGCGCGGCGGCGCTGGCGCCG
GCGCCACGGAAGGAGCCGGGCATGACTGAGAAGAGCGTGCTGCTTTCCGAGCGGCTCATGATATTCAATCTCCTGCCCAG
CCTGACCCTGCATGCCAGTCGCCACGACGAGACGTTTCCAGCCGATTGGGTGCGCGCGTTGTGCAATGCCGACGCGGCGT
TGGCCAACGCATGGCATCGCCATTGGTCGCGCTGGATCTTGTGCGAGCTGGGCCTGCTGAACCAGCCGGTCCTGAGCCTC
GATCCGCCGCAGTTGAAGGTCGCGCTATTGTCCACGGACGCCTTGCGCACCTGCGCCGCCCATGCGGGAGCGCTGCTGTG
CGCGCCGCGCCTGCGACGCGCGATAGACGGCGCTGAGGTCCGTACCTTGCATGCCGCGCTCGGGCGCGATGTGATGAATT
TCGCCGTGTCTTCCGCGGCGCGGGCCCTGCATGACGGGATCGCCGCCAGTTCGGACTGGACCCTGGCCGCCACGGTCCAG
GCGGCGCAGAAACTGGGCTGGGCCGTGCTGCGCGACGCCGTGCAGGGCGCCGCCGACGAGATAGCGCTGCGTTGCGCGCT
GAAGTTGCCGCGCGACCTTGATCCCGCGCCCGTCCTGCCGCCCGAGGCGGCGCTTGCGCTGGTGCTGTCCATGCTCGAAA
TCCTGGATGCCGAATGGCTTTCCTCGTTCCCCGCCCAAGCCTGATCCAGGCGGTACGGCCCGGCCGTGCGGATCCCGCGA
CCGACGTCTTGCGCGCCGAAGACTACGCCGAGCTGCTCAGCGCCGCGCAGATCGTTGCCCAGGCACATCGGCGGGCCGAC
GAAATCGTGGCCGAGGCGCGAGAGGAGTTCGAGCGCGAGCGCAGGCGAGGCTATGAGGAGGGGCGCCGCGAAGCGCTTAC
GGATCAGGCGGAGAAGATGATAGAAACGGTAAGCCGCACGATCGACTACTTCGCGGGTATCGAGAACGAGATGATCGAAC
TGGTCATGAGCGCGGTCCGCAAGAACGTCGACGGTTACGACGACCGCGAGCGCACCGTGATCGCCGTGCGCAACGCATTG
GCGGTCGTGCGCAATCAGCGCCAGATGACCTTGCGCCTGCACCCCGACGAGGTGGATGTGCTCCGGGAAGGCATGAACCA
GCTGCTGGCGGCCTATCCGGGCGTGGGCTACCTGGACCTGCTGCCCGACGCCAGGCTGACGCCGGGAGCCTGCATACTGG
AGAGCGAGATAGGCATGGTCGAGGCCAGCCTCGAGGACCAGCTGTGCGCCTTGCGGGCGGCCTTCGAACGTACATTCGGC
CGGCGCGGATAGGGGCATGCGTCAGTACCACTACATCACGGAGATGATGCGGGTGGCCCTGCAGGATCTGTCCACGTTGC
GGATAAAGGGCCGAGTGGTGCAAGTGGTGGGAACGATCATCAAGGCCGTCGTTCCGATGGTCAAGATCGGCGAAGTGTGC
CTGCTGCGCAATCCTGGCGAGGACTTCGAGATGCACGGCGAAGTGGTGGGCTTTGTTCGCGACGCCGCCTTGCTCACGCC
CATCGGCGACATGTACGGGATTTCCTCGGCGACCGAGGTGATACCGACCGGACGCACGCATATGGTGCCCGTCGGTCCGG
GCTTGCTGGGACGCGTGCTGGACGGGCTGGGACGTCCGCTGGACGTCGCCGAGTCAGGGCCGCTGCATGCCCACAAGTTC
TATCCGGTCTTCGCCGATGCGCCGGATCCGCTGACGCGTCGCATCATCCATGCTCCGCTGGAGCTGGGGGTGCGCGTACT
GGACGGTTTGCTTACATGCGGGGAAGGCCAGCGTCTGGGAATTTTCGCAGCAGCCGGCGGCGGCAAGTCGACCTTGCTGG
GCATGCTGGTCAAGGGCGCCGCGGTCGACGTGACGGTGGTGGCGCTGATCGGCGAGCGTGGGCGGGAAGTTCGCGAGTTC
CTTGAGCACGAACTCGGTCCGGAGGGCAGACGCAAGAGCGTGATCGTTTGCGCGACCAGCGACAAGTCCTCGATGGAGCG
TGCCAAGGCGGCATACGTCGCAACCGCCATCGCCGAATACTTCCGCGATCAAGGGCAGCGTGTACTTTTTCGGATGGATT
CGGTCACCCGCTTTGCGCGAGCCCAGCGTGAAATCGGCTTGGCGGCAGGCGACCCGCCGACGCGGCGCGGCTATCCGCCG
TCGGTGTTCGCCACCCTGCCAAAACTGATGGAGCGCGCCGGCATGAACCAGACGGGTTCGATCACGGCGCTGTATACGGT
GCTGGTCGAGGGGGACGACATGAACGAACCGGTGGCCGACGAGACGCGTTCGATACTGGACGGCCACATCGTGCTCTCGC
GCAAGCTGGGAGCGGCGAATCACTATCCTGCCGTCGACGTCCTGGCCTCAGCCAGCCGGGTCATGAATGCCGTGGTGTCG

CCACGTCACAAGTACCTGGCCGGACGTATGCGCGAACTGATGGCCAAGTACCAGGACGTCGAGCTGTTGGTGAAAATCGG
CGAGTACAAGCAGGGCGCCGATGCGTCGACCGATGAGGCGATACAGAAGATCGGACAGATCAATGCGTTTCTCAGACAAC
TAACCGACGAACGCGAAGCATTCGAGGATACCGTACTGCGCATGGCTGAAATCATCGGACCCGAATCCTAATGGACCTGG
AAAGCCTGCTTGCCATCAAGCATTTTCGCGCCGACCAAGCCCAGCTTGCGCTGAAACGCCAACAGCAGGCCTGCGCGGTT
GCTGCCGCGGCGCAGCGCCAGGCGCAAGGCCGCCTCGACAATTGTCGCCTGTGGGCCGGGCAGCTCGAAAACCGTCTTTA
TGCCGAGCTGTGCCGGCGCATCGTCAAGACACGCGACATCGACGAGGTGCTGCAACGAGTGGGCCACGCCCGCGACCGCC
AGGCCAGCCTGGCGCTGCAGCTCGACAACGCCGTGCGCCGCCACGAGCATGAAATCCAGCTGCTCGCGCAGCAGCGCGAG
CAGCACCGGGAGTGCTTCCAGGCGCAGCAACGGATCGCCGAGTTGGTGCGCCTGCAGCAGGTCGAGGCGGCGGCCTTGCG
CGAGAGCCTGGAGGATCTCTAAATTCAGGAAGCCATCGAATTGTCGGCGCGTGGGCGCGA

Fig. 19

bscV

ATGACGAGCAAGAAATCCATTCTCCGCCTGCAACGCGCGGTGGCGCTGGCCACCAGCCGCAACGACATCGTACTGGCCGT
GCTCATCGTGGCGATCGTCTTCATGATGATCCTGCCGTTGCCCAACCCGACGCTGGTCGACGTGCTGATCGGTGCGAACA
TGACGCTGTCCGCGGTCCTGCTGATGGTCGCGATGTACCTGCCTTCGCCCCTGGCGTTTTCCTCGTTCCCTTCGGTCCTG
CTGGTCACCACGCTGTTCCGGCTGGGCATCTCCATCGCGACCACGCGGCTGATCCTGCTGCAAGGCGATGCCGGCCACAT
CATCGAGACCTTCGGCAACTTCGTGGTGGGCGGCAACCTGATCGTCGGCCTGGTGGTTTTCCTCATCCTCACGATCGTGC
AGTTCGTGGTCATCACCAAAGGCGCGGAGCGGGTGGCCGAAGTCGCCGCGCGCTTCTCGCTGGACGCCATGCCCGGCAAG
CAGATGTCCATCGACGCGGACTTGCGCGCGGGCACCATAGACATGGACGAAGCCCGCCGCCGACGCCGTACGGTCGAGAA
GGAAAGCCAACTGTATGGCGCCATGGACGGCGCGATGAAGTTCGTCAAGGGCGATGCCATCGCCGGCCTGATCATCGTTG
CCGTCAACCTGCTTGGCGGCATGCTGGTCGGCGTGCTGCAGCGCGGCCTGAGCGCCGGCGAGGCCGTGCAGACATATGCC
ATCCTGACCATAGGCGACGGGCTCATCGCGCAGATCCCGGCGCTGTTCATCGCCATCTGCGCGGGAATCATCGTGACGCG
GGTGCAGACCGGGGATGGCCCCTCCAACGTAGGCACCGACATCGGCGCACAAGTGCTGGCGCAGCCTCGCGCCCTGGTCA
TTGCCGGCGCGATCTCGGCAGGCCTGGGCCTCATTCCCGGCATGCCCACGCTGGTCTTCTTCGCCCTGGCCGCCGTGGTG
GGCACCATCGGTTTCGTACTGCTGCGCGCATCCCAGCGTCCGCCCGAAGGCGCCGGGCCCGCGCTCGCCGGCATGGCTGC
CGACGGCCTGCCCCGCACCCGCGCGCCGGCGGATGGACAGGCGGAATTCGCTCCCACCGTCCCGCTGATCATCGACGTGG
CCGCGCGGCTGCAGCCCCGGTTCGAGCCGGCCACCCTCACCGACGATCTGCTGCAGATCCGGCGGGCGCTCTATTTCGAC
TTGGGCGTGCCATTTCCCGGCATCCAGTTGCGCTTCACCGAAGCGCTGGCCGCCAATACCTACACCATCGTGCTGTCGGA
GATCCCGGTGGCGCAGGGGATGTTGCGCGACGATGCCGTGCTGGTGCGGGACACCGAGCAGAACCTGCAGGCCCTGCGGA
TCGCATACGAAACGGGCGCGGCCTTTCTGCCCGATACGCCCACGATCTGGGTTGCAGCCAGTCTGACCGGCGCCTTGCGC
GATGCAGGTATTCCTTACCTGGGTATCAGCCAGATCCTGACTTGGCACTTGGCATATGTACTGAAAAAATATTCAGCCGA
TTTCATCGGCATCCAGGAAACCCGGTTTCTGCTTTCGGCCATGGAAAAACGATTTCCCGATCTGGTCAAGGAGTGCCTGC
GCGTCATGCCGGTGCAGAAGATTGCCGAAATCCTGCAGCGCCTTGTTTCCGAGGAAGTGTCGATACGCAACCTGCGCGCC
GTCCTGGAAGCGCTGGTCGAATGGGGCCAGAAGGAAAAGGATACCGTCCTGCTTACGGAGTATGTCCGAATCGCACTCAA
GCGCTATATCAGCTACAAGTACACCAGCGGCCACAATATCCTGCCCGCCTACCTGCTGGCCCCCAAGGTCGAGGAAACCG
TGCGCGCCGCCATCCGGCAGACCGCCGCCGGCAGTTATTTCGCCCTCGATCCGGACACGACACGCCGGCTGGTCGAGCAC
ATACGCCAATGTGTCGGCGATCTGGCCGCCGGCGCGAGCCGTCCCGTCTTGCTGACGTCGATGGACATCCGGCGCTACAC
GCGCAAGATGATCGAAGCCGATCTCTACGCCCTGCCGGTGCTGTCCTACCAGGAACTGACGCCGGAGATCAATGTACAGC
CCCTGGGCAGGGTGGATCTATGA

bcr3/bscX

Fig. 20

ATGTCCAGCGCCGTACCCGGCATGCATCCCATGCACCTTGGCCTGGAGCGAGGCGTCGACCACATCGTTCCCGGCCCCCG
CTGCGAGCCCGCCCCCACCCTGCCACCCGAGCGCTGGCTCGAGCCGCCCGCCACCGGCGCGGTCGATCATCTGAAAGCCC
TGCTCGTGCGTCCGGACCTGAGCGCGATGCTCGACGAGTCGGCGCGGCCCCGCCTGACGGATGGCGCATTGCTCCAGCCC
GCGCAGTTCGAGCGCGCCCTGGCGCGGGCGCGCGACGAACTGTCCCGGGCCATGGAACTGCACGCCGGCAACACCGCGCC
GGCCTTGAGCCGCGCCTTGCACGTCCTCAACGAGGCCGGAAAGCTGCGTGACCTGGCTGCCATGTATCGCAGCGCGCTCT
ACCAGGGATGA

bopN

Fig. 21

ATGACTCGTATCGATGCCGCCCCCAATCCCTTCCACGCCGCCATGCAGGGGCGCCAGGACGCCTCGGCCAACACTTCCTC

CGGCTGGCTGCAAGGCCAGCGCATCGCACCGGCGCCCACCGGCATATCGCTGGCGGACGCGGCCGAGGAGCTCAGCCTGC
ACATGGCGCAGGCTGCCGAGGAAAAGCATCACTCCGAACGCAAGGTCACGGCCGAACGTCCGATGCTCTGGCTGGACGCG
GCGCAGCTTGCGGAACTGTTTTCCCACACCCACGACCCCGACGCGCAGGCAAAACTGGAAGCCCTGACCGCCGAGCTGCT
GCGCGGCCGGGGCGCCCCCATGCAACTGGCCGCGCAAGCGTTTACCGGTGTCACGCAGCAATACCTCGCGCTGCAGCACG
CGCTGCAGCGCGGCGAGCACGAGGACGCCGCGCCGCACGCGCTCGAAGCCCTGCGCGATGCATTGGCCGACCTGGAGCTC
GCCCATGGCCCCGAAATCCGCGCCGGCATCAACACCCTGCCCACGGCCGGCGCGTTCGCGCGTTCCGCTGACGAGCTGGC
CGGCTTCCAGCACGCGTACCGCGACATCGCCCTGGGCCAGCTGTCGTTGGCGCGCACGCTGGACCTGGTGCTGGAACGCT
ATGGGAACGACGACATCCACGGCGCGCTGGGCGCGCTGATTCAGGCGCTGGGACACGACCTGGCCGCGGCGACACCGTCG
ACGGACGGCGTCAGGCTGCAAGTGTTGGCGAGCGATCTCTATCAAGTCGAGGTGGCCGCCACGGTACTGGAGGAATGCAA
TGCCCTGAAACAACGGCTGGGCAACGCAGGCTCGCAGGAGTGTGCGGACGCCCAGGGCCTGATGCGCGATATTGTGGGAA
TCAGCGAGGACAAATGGATTGCGCCCGCGCGCTTCGAGAAGCTGGCAGAGCGCCACGGCGCGAATGCCCTCTCCGAGCGC
ATCGCATTCCTCGGCGGCGTACGCCAGATTCTCAAAGACCTGCCCACGCAGATCTACGCCGACATGGACGTGCGCGCCAC
CGTCCTGGCGGCCGCGCAGGATGCGCTGGACAACGCGATAGCAATGGAGAACGCATGA

Fig. 22

bsp22

ATGACCATTGATCTCGGAGTTTCACTCACGTCGCAGGCCGGCGGCCTGCAAGGCATCGACCTCAAGAGCATGGATATCCA
GACTCTCATGGTGTATGTGCAGGGTCGTCGCGCCGAACTCCTCACGGCTCAAATGCAGACCCAGGCCGAAGTGGTGCAGA
AGGCCAATGAACGCATGGCGCAGCTCAACGAGGTCCTGTCCGCGCTGTCCCGGGCCAAGGCCGAGTTTCCGCCCAATCCG
AAGCCGGGCGACACCATCCCGGGCTGGGACAACCAGAAGGTCAGCCGGATCGAGGTTCCTCTCAATGATGCGCTGCGCGC
TGCCGGCCTGACGGGCATGTTCGAAGCGCGCGATGGCCAAGTGACCGCCCCCGGCGGCCGGGGTACGCAGGTCGTGAACG
GCACGGGCGTCATGGCCGGTTCCACGACCTATAAGGAACTCGAAAGTGCCTACACCACCGTAAAGGGGATGCTGGATACG
GCGTCCAATACGCAACAGATGGACATGATCAGGCTGCAGGCCGCCAGCAACAAGCGCAACGAGGCTTTCGAGGTCATGAC
CAACACCGAGAAGCGGCGCAGCGACCTGAACAGTTCCATCACCAACAACATGCGCTAA

Fig. 23

bcrH1

ATGCCAAAGTCAGCCGAGCAGGGCGGCTCCCCGGCGTCAGCTTCGCATGAGGCGTTGCGCCATATTCTTGACGCAGGCGC
TTCGATGGGCAGCTTGCAGGGGTTGGACGAGGTGCAACAGCAGGCGTTGTACGCGATCGCTCATGGCGCCTACGAACAGG
GCCGCTATGCCGACGCGTTGAAAATGTTCTGCCTGCTGGTCGCGTGCGATCCGCTGGAAGCCCGTTATCTGCTGGCCCTG
GGCGCCGCGGCCCAGGAGCTGGGGCTGTACGAGCATGCCTTGCAGCAATACGCGGCCGCGGCGGCTTTGCAAGTTGGATT
CCCCCCAGGCCCCTGTTGCATGGCGCCGAGTGCCTGTATGCGTTGGTTCGTCGCCGCGACGCCCTGGATACGTTCGACAT
GGTGCTTGAGTTGTGCGGGTCGCCGGAGCATGCGGCCCTGCGCGAACGGGCCGAGTCGCTGCGCAGGAGCTATGCACGTG
CCGACTGAAACGGCGCCATGTCCGCCGTCAAGATTTCAATTCGAGGAGGTTAGATATGTCTGTTTCTCCGACTTCGCCCG
GCTCTTTCGGGGCCGGCCCTGTCTTTGA

Fig. 24

bopD

ATGTCTGTTTCTCCGACTTCGCCCGGCTCTTTCGGGGCCGGCCCTGTCTTTGACTCCGAATTGCAGGCCCCGGCCCCGTC
GGCGCAGCGTCGCGGCGGTGCGGCGCCTGTGCCGCCGCCCGTCGATCGGCGCGGAGTCGAGCCGGGAGATCCCACGCTGG
GCATGCTGCCCGCGCCCGATTTGCTCGCGGGGGGCGCCGTCAGCCGCACCCGCGCGGCGCTCGACGATCTGGACGCAGCA
CGGCTCGGTGAAGACATCTACACCTTGATGGCGGTGTTGCAACAGGCCAGTCAACAGATGCGGGAGGCCGCCCGTATCGC
TCGTGATGCCGAGGCCACGCGGCAAACGCAGGCTATCGGCGATGCGGCCAGCCAGATGCGCCAGGCGGCCAACGAGCGCA
TGGCCGGAGCGATCGTGGCGGGCGCCATGCAGATAGCGGGTGGTTTCGTGCAGCTGGGGGCGGGCCTGGCAGCGGGTTTG
CAGGCCATGGGTGGCGCAGCTGCGCAAGCCAAGGGCGCCGCATTTTCCGAGCAGGCCTCGACAAGCCGCAAGGTGGCGGC
CGGCTTGCACGATGCCCCCGAGCTGCAGGCAACGGTGCAGGCCCGCGCAACCCAGCTCGAAGCGCAAGCGGCTTCGTTTG
GTGCTGACGCGGCTCGTTCGTCGGCAAAGTCGCAGCGCGTATCGAGCGTTGCCCAGGCCGGCGCCGCGACGGCCGGCGGT
ATCGGCGGCCTGACCAGCGCCGCCCAGGAACGCCGCGCCGCCGAGCACGAGGCCAGGCGCGCGGAGCTGGACGTCGAAGC
GAAGGTGCATGAAACGGCCTCGCGGCGGGCCGACGAAGCCATGCAGCAGATGCTCGACATCATCCGCGGCATCAGGGAAA
AGCTGGCCGGGATGGAGCAGTCCCGCAGCGAGACCGCCCGTAGCGTGGCCCGCAATATCTGA

bopB Fig. 25

ATGACCGTCATGAGTACGACCATATCCACAGCCCCGAGCGGCGCCGCGCTTGCGCCGTCTCGCATAGATATGCGGGCACC
GGAGCCCGGGAGTGCCGGCGAAGGCGCCGGCATCCTGGCGCCGGTGACGACGCTGGCTCTGGCGGCGGGCCGGCCGGCTT
TTCCAGCGTCACCGTCGCTGCGCACCGCGCCCGTCCTGGATCCGCCAGTGCGCGATCTCAGCCCCGCCGACTTGGCCGAC
CTGCTGCGCGTCTTGCGATCCAGGGCGGTGGACGGGCAGTTGGCCACGGCGCGCGAGAACCTGCAGGACGCGCAAGTCAA
GGCGAAGCAGAACACCCAGGCCCAGCTCGACAAGCTGGACGCATGGTTTCGGAAGGCCGAAGAGGCCGAGAGCAAGGGAT
GGCTGAGCAAGGTGTTCGGCTGGATCGGCAAGGTGCTGGCGGTCGTGGCATCGGCCCTGGCGGTGGGCTTTGCCGCCGTC
GCCAGCGTGGCCACCGGCGCGGCGGCCACACCCATGCTGCTGCTCAGCGGCATGGCACTGGTCAGCGCCGTGACATCGCT
GGCCGACCAGATATCGCAAGAAGCGGGAGGCCCGCCTATCAGCCTGGGCGGGTTTCTCTCCGGGCTGGCCGGACGTCTGC
TGACAGCGTTGGGGGTGGATCAGTCGCAGGCCGACCAAATTGCCAAGATCGTCGCCGGCCTGGCCGTGCCCGTCGTCTTG
CTGATCGAACCCCAGATGCTGGGCGAAATGGCGCAAGGCGTGGCCAGGCTGGCTGGCGCCAGCGATGCCACCGCGGGGTA
CATAGCCATGGCGATGTCCATCGTGGCGGCGATCGCGGTCGCCGCGATCAATGCCGCCGGTACAGCCGGCGCGGGTAGCG
CTTCGGCGATCAAGGGGGCCTGGGATCGGGCCGCCGCGGTAGCCACCCAGGTCCTTCAAGGGGGTACGGCAGTGGCGCAA
GGCGGCGTCGGCGTGTCGATGGCAGTCGATCGCAAACAGGCCGATTTCTTGCTCGCCGACAAGGCGGATCTGGCGGCGAG
CCTGACAAAACTGCGGGCGGCCATGGAGCGTGAGGCGGACGATATCAAGAAGATCCTGGCTCAATTCGACGAGGCCTATC
ACATGATCGCGAAGATGATCAGCGATATGGCGAGTACGCACAGCCAGGTCAGCGCCAACCTCGGGCGGCGCCAGGCGGTG
TAG

bcrH2 Fig. 26

ATGACTGTTCACGACGACGCGGCCGCGGCGCTGCGCGCCCGGCTGGATGCGCTGCCGGGCAGCCGGCGCCTGACGGCCGA
GCAATTGGAAGTGATTTACGCGATGGCGTATGCGCACGTCGCCAGGTGCGAGTACGGCAAGGCGCTGCCCATcTTCGCCT
TCCTCGCGCAGTATGGTCCCACGCGCAAGCATTACTGGGCCGGGCTGGCGCTATGCCTGCAGAAGACCGACCGTCCCGAC
GAGGCGCGCAATATCTATGCGTTGATCCTCACGTTCTATCCCGATTCCGCGGACGCCGTGTTGCGCACGGCCGAATGCGA
GCTGGCGTTGGGTGAGAACGAACGGGCACAGGCGGCCCTGTTCGGCGCAATTGCCATCGATGCAGAAAGTGGGCAGCCAG
GTCCGGTCTCGCACCGTGCGCGCGCTTTGCTCGATCTTATTTCAGTTTCACATCCGGAGTAA

bcr4/bscY Fig. 27

ATGGAGCATGTGCTCGAGGAGGCCGACGCCCGCCTGCTTACCGAAGTGGGCTTTCTGGCGGCGGCCGTCAGCGATCTGAC
GCGCGCGGACGCCATTTTCAATGCATTGCAACGTGTACGGCCGGGCCGGACGTATCCCTGCATCGGCCTGGCGGTCGCCC
GCATGAACGCCGGGCTGCCCGACgAAgCCGCCGAgATCCTGGCGAATTTCCAGCCGGCACAGGCGGAGGACCGCTCGGAA
CTGGACGCCTGGTGCGGGTTCGCCCTGTTGCTGGCCGGCCGCTCGGACGAGGCGCGCCGCATGCTGCAGCGAGCCATCGA
TGCGGGTGGCGAGGCGGCAAGGCTGGCGCAGGTCGTGTTGGACAGCGGACCCGCCATGATGCGGCCCGCGCCGTTGCAGT
CCGAGCCATTACCTGGAGCTCCTGGATGA

bscI Fig. 28

ATGAATTTGGATCTGACGGCGATCAACGCCGTGCAGGAACGGCTGCTCGCTCGATCGTTCGACATGCCGCGGTCTCCCGC
GATGGCGGATCAGGCGCGCTTTGAGTTGGCGCTGGGCGAGATGCCCGGCGCATCGGCCCCGAACGGGGCGATCGCCCCGG
CACCGGCCGAAGGCCCGGCGGCGCCGGTCGCGCTCGGCGAGCCGCTGGGCCGCCGCATTCTTGGACAGTTGCGCGGCGGC
CTGGCCGATGTGGCAGGAAAATGGCGGGCGGTGCAGACGGGCTTGGCCGAGGTGAGCCAGGCGCCTACCGTGGTGGGTAT
GCTCGATCTGCAGGCCAGGTTGCTACAGGCATCCGTGGAGTACGAGTTGGTGGGCAAGGCAATAGGGCGCGCCACCCATA
ACGTCTACACGCTGGCGAGAATGTCATGA

bscJ Fig. 29

ATGAACGCCATCGGGGCGATCCAACGGTATCGGCGCGGCGCGGGATGGGCGGCCCTGGCGCTCGCCCTGGCGCTGCTGGC
CGGCTGCGGCGCCCGCGTCGAGCTGTTGGGCGCGGCGCCCGAGAACGAAGCCAACGAAGTATTGGCGGCGCTGCTCGAGG
CAGGCATCGCTGCGCAGAAGCAGTCCGGCAAGGCCGGCTACGCGGTTTCGGTGCCGGCCGAGGCGGTGGCCCGGTCGCTG
GAGATCCTGCGCGCAAGCGGCCTGCCCCGCGAGCAGTTCGACGGAATGGGGCGCATATTCCGCAAGGAAGGCCTGGTTTC
ATCGCCGCTCGAAGAGCGCGCCCGCTACATTTATGCGCTATCTCAGGAATTGGCCGACACCCTGTCGCAGATCGACGGCG
TGCTCAGCGCCCGCGTGCACGTGGTGCTTCCCGAGCGCGGCGCGGTCGGCGAGCCGGCCACCCCTTCGACGGCAGGGGTG
TTTCTCAAGTACCGCGACGGACAGAGCCTCGACGCGCTCGTGCCCGAGATCCGCAAGCTGGTCACGCATGCCATCCCGGG
CCTGGCCGAGGACCGTGTATCGGTTGCCCTGGTGGTGGCCCAGCCCGTTCAGGCCGCACCCGTGCCGGTCGCGTGGCGCC

GCGTGCTTGGCGTACAAGTCGCGGACGGATCGGTCCTGAGATTTTCGCTGTTGCTGCTGTTGTTGCCGGTGCTGTGCCTG
ATAGTGGCGGGGGCCACGCTCTACGCCTGGCGCACGCGCTGGTCCCGCGGCGAAAGGCGCGGCGGCGCTGGCGCCGGCGC
CACGGAAGGAgCCGGGCATGACTGA

bscK Fig. 30

ATGACTGAGAAGAGCGTGCTGCTTTCCGAGCGGCTCATGATATTCAATCTCCTGCCCAGCCTGACCCTGCATGCCAGTCG
CCACGACGAGACGTTTCCAGCCGATTGGGTGCGCGCGTTGTGCAATGCCGACGCGGCGTTGGCCAACGCATGGCATCGCC
ATTGGTCGCGCTGGATCTTGTGCGAGCTGGGCCTGCTGAACCAGCCGGTCCTGAGCCTCGATCCGCCGCAGTTGAAGGTC
GCGCTATTGTCCACGGACGCCTTGCGCACCTGCGCCGCCCATGCGGGAGCGCTGCTGTGCGCGCCGCGCCTGCGACGCGC
GATAGACGGCGCTGAGGTCCGTACCTTGCATGCCGCGCTCGGGCGCGATGTGATGAATTTCGCCGTGTCTTCCGCGGCGC
GGGCCCTGCATGACGGGATCGCCGCCAGTTCGGACTGGACCCTGGCCGCCACGGTCCAGGCGGCGCAGAAACTGGGCTGG
GCCGTGCTGCGCGACGCCGTGCAGGGCGCCGCCGACGAGATAGCGCTGCGTTGCGCGCTGAAGTTGCCGCGCGACCTTGA
TCCCGCGCCCGTCCTGCCGCCCGAGGCGGCGCTTGCGCTGGTGCTGTCCATGCTCGAAATCCTGGATGCCGAATGGCTTT
CCTCGTTCCCCGCCCAAGCCTGA

bscL Fig. 31

ATGGCTTTCCTCGTTCCCCGCCCAAGCCTGATCCAGGCGGTACGGCCCGGCCGTGCGGATCCCGCGACCGACGTCTTGCG
CGCCGAAGACTACGCCGAGCTGCTCAGCGCCGCGCAGATCGTTGCCCAGGCACATCGGCGGGCCGACGAAATCGTGGCCG
AGGCGCGAGAGGAGTTCGAGCGCGAGCGCAGGCGAGGCTATGAGGAGGGGCGCCGCGAAGCGCTTACGGATCAGGCGGAG
AAGATGATAGAAACGGTAAGCCGCACGATCGACTACTTCGCGGGTATCGAGAACGAGATGATCGAACTGGTCATGAGCGC
GGTCCGCAAGAaCGTCGACGGTTACGACGACCGCGAGCGCACCGTGATCGCCGTGCGCAACGCATTGGCGGTCGTGCGCA
ATCAGCGCCAGATGACCTTGCGCCTGCACCCCGACGAGGTGGATGTGCTCCGGGAAGGCATGAACCAGCTGCTGGCGGCC
TATCCGGGCGTGGGCTACCTGGACCTGCTGCCCGACGCCAGGCTGACGCCGGGAGCCTGCATACTGGAGAGCGAGATAGG
CATGGTCGAGGCCAGCCTCGAGGACCAGCTGTGCGCCTTGCGGGCGGCCTTCGAACGTACATTCGGCCGGCGCGGATAG

bscN Fig. 32

ATGCGTCAGTACCACTACATCACGGAGATGATGCGGGTGGCCCTGCAGGATCTGTCCACGTTGCGGATAAAGGGCCGAGT
GGTGCAAGTGGTGGGAACGATCATCAAGGCCGTCGTTCCGATGGTCAAGATCGGCGAAGTGTGCCTGCTGCGCAATCCTG
GCGAGGACTTCGAGATGCACGGCGAAGTGGTGGGCTTTGTTCGCGACGCCGCCTTGCTCACGCCCATCGGCGACATGTAC
GGGATTTCCTCGGCGACCGAGGTGATACCGACCGGACGCACGCATATGGTGCCCGTCGGTCCGGGCTTGCTGGGACGCGT
GCTGGACGGGCTGGGACGTCCGCTGGACGTCGCCGAGTCAGGGCCGCTGCATGCCCACAAGTTCTATCCGGTCTTCGCCG
ATGCGCCGGATCCGCTGACGCGTCGCATCATCCATGCTCCGCTGGAGCTGGGGGTGCGCGTACTGGACGGTTTGCTTACA
TGCGGGGAAGGCCAGCGTCTGGGAATTTTCGCAGCAGCCGGCGGCGGCAAGTCGACCTTGCTGGGCATGCTGGTCAAGGG
CGCCGCGGTCGACGTGACGGTGGTGGCGCTGATCGGCGAGCGTGGGCGGGAAGTTCGCGAGTTCCTTGAGCACGAACTCG
GTCCGGAGGGCAGACGCAAGAGCGTGATCGTTTGCGCGACCAGCGACAAGTCCTCGATGGAGCGTGCCAAGGCGGCATAC
GTCGCAACCGCCATCGCCGAATACTTCCGCGATCAAGGGCAGCGTGTACTTTTTCGGATGGATTCGGTCACCCGCTTTGC
GCGAGCCCAGCGTGAAATCGGCTTGGCGGCAGGCGACCCGCCGACGCGGCGCGGCTATCCGCCGTCGGTGTTCGCCACCC
TGCCAAAACTGATGGAGCGCGCCGGCATGAACCAGACGGGTTCGATCACGGCGCTGTATACGGTGCTGGTCGAGGGGGAC
GACATGAACGAACCGGTGGCCGACGAGACGCGTTCGATACTGGACGGCCACATCGTGCTCTCGCGCAAGCTGGGAGCGGC
GAATCACTATCCTGCCGTCGACGTCCTGGCCTCAGCCAGCCGGGTCATGAATGCCGTGGTGTCGCCACGTCACAAGTACC
TGGCCGGACGTATGCGCGAACTGATGGCCAAGTACCAGGACGTCGAGCTGTTGGTGAAAATCGGCGAGTACAAGCAGGGC
GCCGATGCGTCGACCGATGAGGCGATACAGAAGATCGGACAGATCAATGCGTTTCTCAGACAACTAACCGACGAACGCGA
AGCATTCGAGGATACCGTACTGCGCATGGCTGAAATCATCGGACCCGAATCCTAA

bscO Fig. 33

ATGGACCTGGAAAGCCTGCTTGCCATCAAGCATTTTCGCGCCGACCAAGCCCAGCTTGCGCTGAAACGCCAACAGCAGGC
CTGCGCGGTTGCTGCCGCGGCGCAGCGCCAGGCGCAAGGCCGCCTCGACAATTGTCGCCTGTGGGCCGGGCAGCTCGAAA
ACCGTCTTTATGCCGAGCTGTGCCGGCGCATCGTCAAGACACGCGACATCGACGAGGTGCTGCAACGAGTGGGCCACGCC
CGCGACCGCCAGGCCAGCCTGGCGCTGCAGCTCGACAACGCCGTGCGCCGCCACGAGCATGAAATCCAGCTGCTCGCGCA
GCAGCGCGAGCAGCACCGGGAGTGCTTCCAGGCGCAGCAACGGATCGCCGAGTTGGTGCGCCTGCAGCAGGTCGAGGCGG

CGGCCTTGCGCGAGAGCCTGGAGGATCTCTAA

Table 1

Cytotoxicity of *B. bronchiseptica* towards macrophage-like cell lines in vitro. RB50 (wild type), WD3 *(bscN* deletion) or RB54 (Bvg- phase-locked mutant) were incubated with J774 and RAW macrophage-like cell lines at MOI of 10 for 4 hours. Cytotoxicity is determined by release of lactate dehydrogenase as measured with the CytoTox96 kit and represented as arbitrary units.

	Lactate Dehydrogenase Release (Arbitrary Units)	
B. bronchiseptica Strain	J774	RAW
RB50	853±28 (100%)	1088±31 (100%)
WD3	248±54 (24%)	607±16 (53%)
RB54	110±110 (6%)	51±2 (1%)

Table 2

Sequences of primers used for PCR

For Arbitrary-primed PCR

Name	Sequence (5' to 3')
KB4	CGTACTGCAAGCTTGGTTAACGCGCCGCC
ML2	CATCACGACTGTGCTGGTC

For specific PCR and RT-PCR

Name	Gene specific for	Sequence (5' to 3')
W1	*bscN*	CACAGATCTGGCGAGGACTTCGAGATGCCAC
W2	*bscN*	GAGAATTCCCAGACGCTGGCC
W3	*bscN*	GCGGGGAAGGCCAGCGTCTGGGAATTCTCTC TCGGTCACCCGCTTTGCGGAG
W4	*bscN*	GCACAGATCTCGCCTCATCGGTCGAGCATC
NX7	*fhaB*	CGAACTGACGTCATGGCCGTC
KX31	*fhaB*	GGGGTACCCGCCTGCCCGTGCGCGGTG
BA04	*flaA*	CATCGCCGAGCAGACCGA
BA011	*flaA*	TTGGTACCGACGGCGGCTT

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10690

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/10, 39/02, 39/00; A01N 63/00
US CL :424/240.1, 234.1, 253.1, 254.1, 184.1, 93.2, 93.4; 93.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/240.1, 234.1, 253.1, 254.1, 184.1, 93.2, 93.4; 93.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	YUK et al. The BvgAS virulence control system regulates type III secretion in Bordetella bronchiseptica. Mol. Microbiol. June 1998, Vol. 28, No. 5, pages 945-959, see entire document.	1-20 and 47
Y	WOESTYN et al. YScN, the putative energizer of the Yersinia Yop secretion machinery. J. Bacteriol. March 1994, Vol. 176, No. 6, pages 1561-1569, see entire document.	1-10
Y, P	EP 0 889 120 A1 (IMPERIAL COLLEGE INNOVATIONS LIMITED) 07 January 1999, see entire document.	1-20 and 47

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 24 SEPTEMBER 1999	Date of mailing of the international search report 22 OCT 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer S. DEVI JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATTERS Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10690

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HENSEL et al. Functional analysis of ssaJ and the ssaK/U operon, 13 genes encoding components of the type III secretion apparatus of Salmonella Pathogenicity Island 2. Mol. Microbiol. 1997, Vol. 24, No. 1, pages 155-167, see entire document.	1-10
Y	BOLAND et al. Role of YopP in suppression of tumor necrosis factor alpha release by macrophages during Yersinia infection. Infect. Immun. May 1998, Vol. 66, No. 5, pages 1878-1884, see entire document.	1-10
A	COTTER et al. BvgAS-mediated signal transduction: Analysis of phase-locked regulatory mutants of Bordetella bronchiseptica in a rabbit model. Infect. Immun. August 1994, Vol. 62, No. 8, pages 3381-3390, see entire abstract.	1-20 and 47
A	COTTER et al. A mutation in the Bordetella bronchiseptica bvgS gene results in reduced virulence and increased resistance to starvation, and identifies a new class of Bvg-regulated antigens. Mol. Microbiol. 1997, Vol. 24, No. 4, pages 671-685, see entire document.	1-20 and 47
A	TEJADA et al. Comparative analysis of the virulence control systems of Bordetella pertussis and Bordetella bronchiseptica. Mol. Microbiol. 1996, Vol. 22, No. 5, pages 895-908, see entire document.	1-20 and 47
A	LEE CA. Type III secretion systems: machines to deliver bacterial proteins into eukaryotic cells? Trend. Microbiol. April 1997, Vol. 5, No. 4, pages 148-156, see entire document.	1-20 and 47
A, P	GALAN et al. Type III secretion machines: Bacterial devices for protein delivery into host cells. Science, 21 May 1999, Vol. 284, pages 1322-1328, see entire document.	1-20 and 47

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10690

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-20 and 47

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG, MEDLINE, EMBASE, BIOSIS, TOXLINE, AGRICOLA, FEDRIP, PASCAL, JAPIO, DERWENT, JICTEPLUS, Inside Conferences and Dissertation Abstracts.
Serach terms: Bordetella, type III secretion mutant, bscN, bsp22, inventors' names

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claim(s) 1-20 and 47(1), drawn to a genetically engineered secretion system *Bordetella* mutant, a vaccine comprising the same and a method for vaccinating using the mutant.
Group II, claim(s) 40-46 and 47(2) (i.e. second claim numbered 47), drawn to a live mucosal antigen delivery vector, a vaccine comprising the same and a method for vaccinating using the same.
Group III, claim(s) 21-39 and 48-51, drawn to a purified nucleic acid comprising a sequence encoding a *Bordetella* type III secretion system component, a polypeptide encoded by a nucleic acid, a DNA molecule encoding a polypeptide and a method for cloning a DNA molecule.
The inventions listed as Groups I, II and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of invention I is a genetically engineered type III secretion system *Bordetella* mutant, a line attenuated vaccine component against *Bordetella* comprising the mutant and a method for vaccinating an animal by administering the mutant. Individually, the product(s) and the first method of use of the product(s) are a permitted combination of categories under PCT Rule 13.2. Invention II is directed to a second product and a method for its use, i.e., a live mucosal antigen-delivery vector, a vaccine comprising the same and a method of immunization. Invention III is directed to a third product, a purified nucleic acid comprising a sequence, a polypeptide encoded by the nucleic acid sequence and a method of cloning a DNA molecule. The three products are structurally, functionally and biologically distinct. The special technical features of the inventions are not linked and therefore the inventions lack unity.

CORRECTED VERSION*

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 39/10, 39/02, 39/00, A01N 63/00

A1

(11) International Publication Number: WO 99/59630

(43) International Publication Date: 25 November 1999 (25.11.99)

(21) International Application Number: PCT/US99/10690

(22) International Filing Date: 14 May 1999 (14.05.99)

(30) Priority Data:
60/085,691 15 May 1998 (15.05.98) US

(71) Applicant *(for all designated States except US)*: UNIVERSITY OF CALIFORNIA LOS ANGELES [US/US]; Office of Technology Transfer, 5th floor, 1111 Franklin Street, Oakland, CA 94607–5200 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MILLER, Jeff, F. [US/US]; 3469 Alana Drive, Sherman Oaks, CA 91403–4705 (US). HARVILL, Eric, T. [US/US]; 2491 Purdue Avenue #119, Los Angeles, CA 90064 (US). YUK, Ming, H. [SG/US]; 11811 Venice Boulevard #119, Los Angeles, CA 90066 (US). COTTER, Peggy, A. [US/US]; 20658 Martinez Street, Woodland Hills, CA 91364–2310 (US).

(74) Agents: WISE, Michael, J. et al.; Lyon & Lyon, Suite 4700, 633 West 5th Street, Los Angeles, CA 90071–2066 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: TYPE III *BORDETELLA* SECRETION SYSTEM

(57) Abstract

The invention relates to a *Bordetella* type III secretion system and its constituent components, genetically modified *Bordetella*, prophylactic and remedial live attenuated vaccines including genetically modified *Bordetella* and uses thereof.

*(Referred to in PCT Gazette No. 16/2000, Section II)

BNSDOCID: <WO___9959630A1_IA>

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TYPE III *BORDETELLA* SECRETION SYSTEM

This application claims benefit under 35 U.S.C. § 119(e) to United States Provisional Application No. 60/085,691 filed May 15, 1998, incorporated herein by reference.

FIELD OF THE INVENTION

The invention relates to a *Bordetella* type III secretion system and its constituent components, genetically modified *Bordetella*, prophylactic and remedial live attenuated vaccines including genetically modified *Bordetella* and uses thereof.

BACKGROUND OF THE INVENTION

The genus *Bordetella* includes small, aerobic, Gram-negative coccobacilli associated with respiratory infections in humans and other animals. *B. pertussis* infects humans and causes whooping cough, a highly contagious disease with severe clinical manifestations in children (Hewlett, 1995). (Throughout this application various publications are referenced, the disclosures of these publications in their entireties are hereby incorporated by reference.) *B. parapertussis* causes a similar disease in humans and has also been recently isolated from sheep (Cullinane et al., 1987; Porter et al., 1994). *B. bronchiseptica* infects a broad range of mammals, and has been isolated from mice, rats, guinea pigs, rabbits, skunks, opossums, raccoons, cats, dogs, ferrets, foxes, pigs, hedgehogs, sheep, koala, bears, leopards and horses. Although bordetellosis is often asymptomatic, *B. bronchiseptica* can and does cause significant disease.

B. bronchiseptica and *Pasteurella multocida*, are a primary cause of infectious atrophic rhinitis in swine, a serious and widespread disease responsible for significant economic loss to the pork industry. Atrophic rhinitis is an upper respiratory disease that results in degeneration of the nasal turbinates, deviation of the nasal septum and atrophy of the nasal bone, which can be so severe that it causes visible deformation of the animal's snout. Pigs suffering from atrophic rhinitis feed less robustly and gain weight more slowly than healthy pigs, resulting in lower market weight and/or longer time-to-market. Atrophic rhinitis affects pigs worldwide causing significant global economic impact on the pork-producing industry.

Atrophic rhinitis is caused by co-infection of the swine upper respiratory tract with *B. bronchiseptica* and *Pasteurella multocida*. Results from both *in vivo* and *in vitro* studies indicate that colonization of the upper respiratory epithelium with *B. bronchiseptica* predisposes pigs to infection by the opportunistic *P. multocida* which would otherwise be unable to efficiently establish infection. Although colonization by *B. bronchiseptica* alone may lead to pronounced turbinate atrophy, disease is most severe, sometimes progressing to pneumonia, when exacerbated by secondary infection with *P. multocida*. The inability of *P. multocida* to efficiently infect *Bordetella*-free swine suggests preventing infection with *Bordetella* would also prevent infection by *P. multocida* and the resulting atrophic rhinitis.

B. bronchiseptica is also responsible for causing infectious tracheobronchitis (ITB) (kennel cough) in dogs and a similar respiratory illness in cats. ITB has a worldwide distribution and commonly develops in dogs that are housed in groups. Because of its highly infectious nature, ITB is a major concern for breeding, boarding and training kennels as well as animal care facilities at research and training institutions. Although most facilities require dogs to be vaccinated against *B. bronchiseptica*, the efficacy of currently available vaccines is questionable at best and ITB remains a common and important canine disease. *B. bronchiseptica* also causes an upper respiratory disease in rabbits called snuffles. As with atrophic rhinitis, snuffles is exacerbated by co-infection with *P. multocida* and can progress to bronchopneumonia. Rats and mice are also natural hosts for *B. bronchiseptica* and are highly susceptible. The ID_{50} for infection with *B. bronchiseptica* in many cases is less than 20 cfu.

Bordetella spp. produce a number of protein factors (*e.g.*, virulence factors) that play important roles in the interaction between the bacteria and host cells, leading to establishment of infection, pathogenesis and transmission (Weiss and Hewlett, 1986). Protein factors involved in adhesion to host cells include filamentous hemagglutinin (FHA), pertactin and fimbriae (Mooi et al., 1992. Relman et al., 1989; Roberts et al., 1991); while production of toxins, including the bifunctional adenylate cyclase toxin/hemolysin (AC/HLY) (Hewlett and Gordon, 1988), dermonecrotic toxin (Walker and Weiss, 1994) and pertussis toxin (expressed only in B. pertussis) (Locht et al., 1986), are involved in the pathological effects on host tissues and the ability to evade host defenses.

Expression of these virulence factors is coordinately regulated by the bvgAS locus. BvgA and BvgS, which belong to the family of two-component signal transduction proteins

found in prokaryotes and lower eukaryotes, are responsible for sensing external stimuli and coordinating the transcription of a large collection of genes and operons that function during the infectious cycle (Stibitz et al., 1988; Stibitz and Yang, 1991). Signal transduction is accomplished by a His-Asp-His-Asp phosphorelay mechanism (Uhl and Miller, 1994; Uhl and Miller, 1996; Uhl and Miller, 1996).

The Bvg^+ phase is characterized by expression of virulence factors and this phase is necessary and sufficient for colonization of rabbits and rats (Akerley et al., 1995; Cotter and Miller, 1994). The Bvg^- phase is avirulent and characterized by the loss of virulence gene expression and induction of genes which are repressed in the Bvg^+ phase, including motility genes in *B. bronchiseptica* (Akerley and Miller, 1993; Akerley et al., 1992).

There is a need for an effective *B. bronchiseptica* vaccine. Live attenuated vaccines, i.e., living viruses and bacteria that carry mutations rendering them avirulent or greatly reduced in virulence offer significant advantages in terms of manufacture and immunogenicity. A single inoculation of live vaccine at a modest dose may replicate *in vivo* to a large immunogenic dose and, during the course of replication, express the majority of immunogens seen during natural disease. The processing and presentation of these antigens more closely corresponds to that of infection. Live attenuated vaccines can induce mucosal immune responses, which are not efficiently elicited by systemically administered vaccines. The mode of delivery of the live attenuated vaccines is more simple, requiring ingestion or inhalation. Finally, because attenuated vaccines are living organisms, they may also be used as vectors by genetically engineering them to express heterologous antigens, thus providing a mechanism for protection from more than one disease.

Most infectious agents either infect mucosal surfaces directly or gain entry to the body via mucosae or mucosal lymphoid tissue. Immunization that elicits mucosal antibody is therefore a cost effective tool to prevent and combat infectious diseases. Mucosal immunity is believed to be dependent on the production of an IgA antibody response, which generally cannot be accomplished by conventional vaccine administration routes, for example injection of the antigen.

Live attenuated bacterial vaccines that generate effective mucosal immune responses have been successfully derived from modified bacteria of the genera *Mycobacterium*, *Salmonella*, *Shigella*, *Vibrio* and *Listeria*. The progress in the field over the last decade is reviewed in Killeen, et al., *Bacterial mucosal vaccines: Vibrio cholerae as a live attenuated*

vaccine/vector paradigm, Curr Top Microbiol Immunol. 236:237-54 (1999). The goal of eliciting a mucosal immune response, in contrast to the elicitation of a systemic immune response in the absence of a mucosal immune response, is in principle necessary because the immune system is organized such that injected antigens are not effective in eliciting the generation of antibodies in what is known as the "common mucosal immune system."

The common mucosal immune system is an integrated system including gut-associated lymphoid tissue and nasal-associated lymphoid tissue. The common mucosal immune system is stimulated by antigens entering central inductive sites present in mucosal tissues, where they interact with accessory and lymphoid cells and stimulate specific IgA-committed B cells and appropriate T cells. These cells then migrate to effector sites, including the lamina propria of the gastrointestinal and respiratory tracts as well as to remote effector sites, such as the exocrine glands, including the salivary, mammary and lacrimal glands. At the effector sites, surface IgA+ B cells clonally expand and terminally differentiate into IgA-secreting plasma cells under the influence of antigen, T helper cells and cytokines, such as interleukin (IL)-5, IL-6, and IL-10.

Live attenuated strains of pathogenic bacteria have proven uniquely successful in generating protective immune responses against the wild type pathogenic bacteria at mucosal sites. The live attenuated strains have also proven very effective in generating protective immune responses against other pathogens, particularly when the live attenuated bacteria are genetically engineered to express antigenic epitopes of these other pathogens.

Two modes of attenuation have been used: undefined mutations and defined mutations. The most widely used attenuated bacterial vaccines - *Salmonella typhi* strain Ty21a and *Mycobacterium bovis* strain Bacille, Calmette, Guerin (BCG) - were attenuated by undefined mutations. BCG is widely used throughout the world as a live vaccine for tuberculosis and in the treatment of certain cancers. *S. typhi* Ty21a is administered in three or four oral doses and is very well tolerated in humans. The immune response elicited is both humoral and cell-mediated and confers significant, but incomplete, protection from typhoid fever.

Numerous attenuated strains of *Vibrio*, *Salmonella*, and *Shigella* that carry defined attenuating mutations have also been generated. These defined genetic mutations have been of two subclasses (1) disruption of gene(s) affecting metabolism or regulation and (2) disruption of gene(s) affecting virulence. While the metabolically attenuated strains proved

unsuccessful either due to their inability to provoke significant immune responses or because they caused unacceptable levels of high fever and bacteremia, virulence attenuation has proven to be a highly successful strategy. Killeen, et al., at 240-243. Safer and more effective vaccines have thus been constructed by virulence attenuation.

Moreover, the successful genetic attenuation of live bacteria resulting in safe, immunogenic and protective vaccines has enabled the development of safe vaccine vectors. The use of genetically attenuated live bacteria as vaccine vectors has proven successful against viral, bacterial and parasitic derived pathogens. BCG, for example, has vectored several bacterial and viral antigens from Borrelia, pneumococci, simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV), eliciting immune responses against each antigen when used to immunize mice. BCG vectored outer-surface protein A (OspA) and pneumococcal-surface protein A (PspA) conferred protection against *Borrelia* and *S. pneumoniae*, respectively in murine challenge models.

Attenuated *Salmonella* have proven capable of delivering more than 30 bacterial, 10 viral and 10 parasitic antigens in pre-clinical murine studies. Kaufmann, Concepts in vaccine development, de Gruyter, Berlin (1996). Protective immunity was demonstrated with bacterial challenge with *Y. pestis*, *L. monocytogenes* and *B. pertussis*, viral challenge with Herpes simplex virus and influenza, parasitic challenge by *L. major* and *S. mansoni*, among others. Kaufmann, Concepts in vaccine development, de Gruyter, Berlin (1996).

Listeria monocytogenes has also been successfully used as a live attenuated vaccine vector. *See, e.g.*, Shen H, et al., *Compartmentalization of bacterial antigens: differential effects on priming of CD8 T cells and protective immunity*, Cell. 92(4):535-45 (1998); Jensen ER, et al., *Recombinant Listeria monocytogenes vaccination eliminates papillomavirus-induced tumors and prevents papilloma formation from viral DNA*, J Virol. 71(11):8467-74 (1997); Jensen ER, et al., *Recombinant Listeria monocytogenes as a live vaccine vehicle and a probe for studying cell-mediated immunity*, Immunol Rev. 158:147-57 (1997); Slifka MK, et al., *Antiviral cytotoxic T-cell memory by vaccination with recombinant Listeria monocytogenes*, J Virol. 70(5):2902-10 (1996); Shen H, et al., *Recombinant Listeria monocytogenes as a live vaccine vehicle for the induction of protective anti-viral cell-mediated immunity*, Proc Natl Acad Sci U S A. 92(9):3987-91 (1995).

SUMMARY OF THE INVENTION

In a first, independent aspect of the present invention, a *Bordetella* type III secretion system and its constituent components is disclosed.

In a second, independent aspect of the present invention, genetically engineered *Bordetella* having modifications of the type III secretion system and its constituent components is disclosed.

In a third, independent aspect of the present invention, live genetically attenuated *Bordetella bronchiseptica* having modifications of the type III secretion system or its constituent components effective in preventing infection with wild type *Bordetella bronchiseptica* is disclosed.

In a fourth, independent aspect of the present invention, a vaccine including genetically engineered *Bordetella bronchiseptica* having modifications of the type III secretion system and/or its constituent components effective in preventing infection with wild type *Bordetella bronchiseptica* is disclosed.

In a fifth, independent aspect of the present invention, methods are disclosed for using genetically engineered *Bordetella bronchiseptica* having modifications of the type III secretion system as a vaccine against wild type *Bordetella bronchiseptica.*

In a sixth, independent aspect of the present invention, genetically engineered, attenuated *Bordetella* expressing a heterologous antigen effective as a live mucosal antigen delivery vector is disclosed.

In a seventh, independent aspect of the present invention, genetically engineered *Bordetella* expressing a heterologous antigen effective in generating a mucosal immune response to the heterologous antigen is disclosed.

In an eighth, independent aspect of the present invention, a vaccine including *Bordetella* expressing a heterologous protective antigen effective in generating a mucosal immune response to the heterologous antigen is disclosed.

In a ninth, independent aspect of the present invention, methods are disclosed for using *Bordetella* expressing a heterologous antigen to generate a mucosal immune response to the heterologous antigen.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Differential display PCR with primers KB4 and ML2 (see Table 2) identified fragments produced only from the Bvg^+ but not Bvg^- phase cDNA. cDNAs made from

total RNA of *B. bronchiseptica* strain RB50 grown in the Bvg^+ phase (lanes 1, 2, 5, 6) or Bvg^- phase (lanes 3, 4, 7, 8) were used as templates for PCR with primer KB4 (lanes 1 to 4) or primer ML2 (lanes 5 to 8). Each reaction was done in duplicate except that the odd numbered lanes contain twice the amount of cDNA template as the even numbered lanes. Arrows indicate positions of prominent bands amplified only from Bvg^+ phase cDNA.

Figure 2: Protein sequence of BscN compared to YscN. The two conserved Walker Boxes are outlined, and the shaded region is deleted in the mutant strain WD3.

Figure 3. Genomic organization and open reading frames (arrows) of type III secretion genes in *B. bronchiseptica*. The numbers below each open reading frame indicate the percentage amino acid identity to corresponding homologues in (1) *Yersinia spp.* and (2) *Pseudomonas aeruginosa*. The letters A, B and C denote ORFs of secreted proteins identified in figure 5.

Figure 4: *BscN* expression is regulated by *bvg* and it is expressed in the Bvg^+ phase. a. RT-PCR with primers specific for *fhaB*, *fla*A and *bscN*. Genomic DNA (g) and cDNAs made from total RNA of *B. bronchiseptica* strain RB50 grown in Bvg^+ phase (+) or Bvg^- phase (-) were used as templates for PCR. Controls of mock RT were templates in which the RT reactions were done in the absence of reverse-transcriptase. Primer pairs specific for *fhaB* (NX7+KX3l), *flaA* (BA04+BA011) and two separate regions of *bscN* (W1+W2 and W3+W4) were used for PCR. See Table 2 for sequences of primers. b. RT-PCR with the same primers as in part a, but with genomic (g), cDNAs and mock RT templates from phase-locked strains of *B. bronchiseptica* RB53 (locked in Bvg^+ phase) and RB54 (locked in Bvg^- phase). c. Slot blots of total RNA with probes for *fhaB*, *flaA*, *recA* and *bscN*. ^{32}P labeled DNA probes were used to detect specific transcripts in total RNA isolated from RB50 grown in Bvg^+ phase (+) or Bvg^- phase (-). RNA samples were diluted two-fold and eight-fold in the second and third rows respectively of each panel.

Figure 5: In-frame deletion of *bscN* causes decreased secretion of certain polypeptides from *B. bronchiseptica in vitro*. TCA precipitates of proteins from culture supernatants of wild type strain RB50 (lanes 1, 3, 6, 8, 10, 12), *bscN* deletion strain WD3 (lanes 2, 4, 7, 9, 11, 13) and Bvg^- phased-locked strain RB54 (lane 5) were separated by 4 to 12% gradient SDS-PAGE and detected by: Coomassie stain (lanes 1 and 2); immunoblots with antisera from rabbit (lanes 3 to 5), rat (lanes 6, 7, 10, 11) and mouse (lanes 8, 9, 12, 13), which were previously infected with RB50 (lanes 3 to 9) or WD3 (lanes 10 to 13). Open

arrows indicate major proteins produced from RB50 but not WD3 as detected by Coomassie staining while solid arrows indicate major proteins produced only from RB50 as detected by immunoblots. Numbers on the right column indicate positions of molecular weight markers in kiloDaltons. Figure 5' is another reproduction of the photograph in figure 5. Polypeptides indicated as A, B and C were electroblotted onto PVDF paper and their amino-terminal sequences determined by Edman degradation. Amino terminal sequence of A was determined as: XRIDAARNPXHAAMQ. Amino terminal sequence of B was determined as: SVSPTSPGSFGAGPV. Amino terminal sequence of C was determined as: TIDLGVSLTSQAGGL. Three ORFs that correspond to these amino-terminal sequences are indicated in figure 3.

Figure 6: Deletion of *bscN* leads to decreased cytotoxicity of *B. bronchiseptica* on L2 epithelial cells. L2 cells were incubated with *B. bronchiseptica* strains RB50 (b and c), WD3 (e and f) and FF1 (d) with MOI of 500 or without any bacteria (a) and incubated for 15 minutes (b and e) or two hours (c, d and f). Cells were washed, fixed, stained with Giemsa stain, and observed under microscope.

Figure 7: Deletion of *bscN* eliminates tyrosine dephosphorylation of certain proteins in L2 cells upon infection with *B. bronchiseptica*. Confluent L2 cells were incubated with *B. bronchiseptica* strains RB50 (lanes 3 and 4), WD3 (lane 5) and FF1 (lane 6) with MOI of 500 or without any bacteria (lane 2) for 1 hour. 1 mM vanadate was added to one sample of L2 cells incubated with RB50 (lane 4). Total cell protein from RB50 itself (lane 1) and each of the above samples of infected L2 cells were separated by 8% SDS-PAGE and probed with monoclonal antibody PT-66 specific for phosphotyrosine. Arrows indicate tyrosine-phosphorylated proteins that are apparently dephosphorylated upon incubation with RB50, but not with WD3 or FF1.

Figure 8: Deletion of *bscN* causes defect in persistence of *B. bronchiseptica* in the trachea of rats. Female 4 week old Wistar rats were inoculated intranasally with 1000 cfu of either RB50 or WD3 in a 5µl suspension. Groups of 4 animals were sacrificed 14 and 35 days post-inoculation. The nasal septum and 1 cm of the trachea were homogenized and plated on BG agar to determine recoverable cfu of *B. bronchiseptica* from these tissues of each animal. Differences between cfus of RB50 and WD3 recovered from the nasal septum on days 14 and 35, or from the trachea at day 14, were not statistically significant. Dotted line indicates the minimum level of detection.

Figure 9: *BscN* is not transcribed in most *B. pertussis* strains and a human isolate of *B. parapertussis*. Genomic DNA (g) and cDNAs from total RNA of Bvg^+ phase (+) and Bvg^- phase (-) cells of the following strains were used as templates for PCR: *B. bronchiseptica* RB50; *B. pertussis* strains GMT1, 17471, Tohama 1, 18323; human isolate of *B. parapertussis* strain A168 and ovine isolate of *B. parapertussis* strain Hl. Primers specific for *bscN* in RB50 (W3 + W4) were used for PCR of the genomic DNA, cDNAs and also mock RT samples (0).

Figure 10. In-frame deletion of *bsp22* does not affect secretion of other type III secreted polypeptides. TCA precipitates of 1 ml of culture supernatant of wild type (RB50), *ΔbscN* (WD3) and *Δbsp22* (D218) strains of *B. bronchiseptica* were immunoblotted with mouse antiserum specific for Bsp22 (A) antisera from a rabbit previously infected with RB50 (B). Thick arrow indicates Bsp22, thin arrows indicate other as yet unidentified type III secreted polypeptides, which are not secreted by WD3. Bsp22 secretion is restored in D218 by the presence of plasmid pLB2, which contains the ORF of *bsp22* in the broad host range vector pBBR1MCS (A, right lane).

Figure 11. Disruption of type III secretion leads to more rapid clearance of *B. bronchiseptica* from the trachea of C57BL/6 mice (A) and reduced colonization of the trachea of BALB/c mice (B). Female 4 week old mice were inoculated intranasally with 10^5 cfu of wild type (RB50), *ΔbscN* (WD3) or *Δbsp22* (D218) strains in a 50 μl suspension. Groups of 3 to 4 animals were sacrificed 7 and 35 days post- inoculation. The nasal extract and 0.5 cm of the trachea of each animal were homogenized and plated on BG agar to determine recoverable cfu of *B. bronchiseptica* from these tissues of each animal. Dotted line indicates the minimum level of detection. Horizontal bars indicate means of cfu counts. Recovered cfu from the trachea showed significant difference (*) between that of RB50 and WD3 infected BALB/c mice on day 35 ($p=0.03$). Recovered cfu from the trachea also showed significant difference (*) between that of RB50 and D218 infected BALB/c mice on day 35 ($p=0.01$).

Figure 12. Type III secretion mutants elicit higher titers of anti-*Bordetella* antibodies in infected mice compared to wild type infected animals. Wild type bacterial cells were coated onto ELISA plates and probed with serial dilutions of sera from BALB/c (A) or C57BL/6 (B) mice infected with RB50 or WD3 or D218 for 35 days. Bound anti-*Bordetella* antibodies were detected by secondary antibodies specific for total immuno-

globulins. Results were averages from 3 or 4 animals in each group. * indicates statistically significant difference in titers between serum from wild type infected host and that from type III secretion mutant infected host at $p<0.05$.

Figure 13. Disruption of type III secretion causes increase in virulence of *B. bronchiseptica* in SCID-beige mice. (A) 500 cfu of RB50, WD3, D218 or RB54 (*Δbvg*) in 5 µl droplets were inoculated into the nasal cavity of groups of 10 SCID-beige mice. Health of the mice was monitored over a three month period and morbid mice were sacrificed prior to death. Time of survival of the mice inoculated with RB50 (circles) were significantly (as determined by Logrank analysis) longer than those infected with WD3 (triangles) (Chi-square= 9.701, P-value=0.0018) or D218 (squares) (Chi-square= 9.605, P-value=0.0019). (B) Growth curve of RB50, WD3 and D218 in Stainer-Scholte medium at 37°C with aeration as determined by optical density of liquid culture. (C) Similar numbers of bacteria could be recovered from SCID-beige mice infected with wild type or type III secretion defective strains of *B. bronchiseptica* in early stages of infection. $5x10^5$ cfu of RB50, WD3 or D218 were inoculated into groups of 3 to 4 SCID-beige mice and animals were sacrificed after 5 days. Recoverable cfu from the nasal cavities and lungs of animals were determined and compared. Unpaired t-tests showed no significant differences in the recovered cfu from each organ among different bacterial strains.

Figure 14. Cytotoxicity of *B. bronchiseptica* towards macrophage-like cell lines *in vitro* depends mostly on type III secretion. RB50 (wild type), WD3 (*ΔbscN*), D218 (*Δbsp22*) or RB54 (Bvg$^-$ phase-locked mutant) were incubated with J774A.1 macrophage-like cell lines at MOI of 10 for 2 hours. Cytotoxicity is determined by release of lactate dehydrogenase as measured with the CytoTox96 kit and represented as arbitrary units.

Figure 15. Type III secretion is required for induction of apoptosis of J774A.1 mouse macrophage cell lines by *B. bronchiseptica*. J774A.1 cells were infected with wild type strain RB50 (A), *ΔbscN* strain WD3 (B), or *Δbsp22* strain D218 (C) with MOI of 50 for 60 minutes, then washed, fixed, permeabilized and incubated with fluorescent TUNEL reagent according to manufacturer's protocol. Each sample was observed by differential interference contrast microscopy (top row) or epifluorescent microscopy (bottom row).

Figure 16. Type III secretion is required for induction of apoptosis by *B. bronchiseptica in vivo*. Female 4 week old BALB/c mice were inoculated intranasally with 10^5 cfti of PBS (A), RB50 (B) or WD3 (C) in a 50 µl suspension. The animals were

sacrificed after 2 days and their lungs were fixed and sectioned as described in experimental procedures. The sections were then deparaffinized, labeled with fluorescent TUNEL reagent according to manufacturer's protocol and observed under phase-contrast epifluorescent microscope. Images are overlays of phase-contrast and fluorescent images of the same view.

Figure 17. Type III secretion by *B. bronchiseptica* is required for aggregation and inactivation of *NF-κB* in L2 cells *in vitro*. The rat lung epithelial cell line L2 was incubated with: (A) medium alone; (B) medium with 10 ng/mlTNFα for 10 minutes; (C) wild type *B. bronchiseptica* (RB50) at MOI of 100 for 20 minutes; (D) wild type *B. bronchiseptica* (RB50) at MOI of 100 for 20 minutes, followed by 10 ng/ml TNFα for 10 minutes; (E) *ΔbscN* strain of *B. bronchiseptica* (WD3) at MOI of 100 for 20 minutes; (F) *ΔbscN* strain of *B. bronchiseptica* (WD3) at MOI of 100 for 20 minutes, followed by 10 ng/ml TNFα for 10 minutes. Cells were fixed, permeabilized, labeled with antibody specific for *NF-κB* p65 subunit and observed by epifluorescent microscopy as described in materials and methods.

Figure 18. DNA sequence corresponding to figure 3. Seq. ID 1.

Figure 19. DNA sequence corresponding to ORF of bscV. Seq. ID 2.

Figure 20. DNA sequence corresponding to ORF of bcr3/bscX. Seq. ID 3.

Figure 21. DNA sequence corresponding to ORF of bopN. Seq. ID 4.

Figure 22. DNA sequence corresponding to ORF of bsp22. Seq. ID 5.

Figure 23. DNA sequence corresponding to ORF of bcrH1. Seq. ID 6.

Figure 24. DNA sequence corresponding to ORF of bopD. Seq. ID 7.

Figure 25. DNA sequence corresponding to ORF of bopB. Seq. ID 8.

Figure 26. DNA sequence corresponding to ORF of bcrH2. Seq. ID 9.

Figure 27. DNA sequence corresponding to ORF of bcr4/bscY. Seq. ID 10.

Figure 28. DNA sequence corresponding to ORF of bscI. Seq. ID 11.

Figure 29. DNA sequence corresponding to ORF of bscJ. Seq. ID 12.

Figure 30. DNA sequence corresponding to ORF of bscK. Seq. ID 13.

Figure 31. DNA sequence corresponding to ORF of bscL. Seq. ID 14.

Figure 32. DNA sequence corresponding to ORF of bscN. Seq. ID 15.

Figure 33. DNA sequence corresponding to ORF of bscO. Seq. ID 16.

DETAILED DESCRIPTION OF THE INVENTION

In one embodiment, the invention provides a type III secretion system in *Bordetella*. This system comprises multiple proteins encoded by nucleic acid sequences that comprise the type III secretion system for *Bordetella*. In one embodiment, the DNA sequence of Fig. 18 is provided. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bscV corresponding to Figure 19. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bcr3/bscX corresponding to Figure 20. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bopN corresponding to Figure 21. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bsp22 corresponding to Figure 22. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bcrH1 corresponding to Figure 23. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bopD corresponding to Figure 24. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bopB corresponding to Figure 25. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bcrH2. corresponding to Figure 26. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bcr4/bscY corresponding to Figure 27. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bscI corresponding to Figure 28. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bscJ corresponding to Figure 29. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bscK corresponding to Figure 30. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bscL corresponding to Figure 31. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bscN corresponding to Figure 32. In another embodiment, one protein of the type III secretion system is encoded by a nucleic acid sequence designated bscO corresponding to Figure 33.

Further, nucleic acid sequences are provided that hybridize (e.g., under stringent conditions) to the nucleic acid sequences of the invention, including, for example, the

homologous genes in *B. pertussis* and *B. parapertussis*. Additionally, the invention provides polypeptides encoded by the nucleic acid molecules of the invention.

Bordetella spp. attenuated in accordance with the present invention are effective bacterial vectors for immunization against *Bordetella* infection as well as against heterologous antigens.

In accordance with the present invention, Type III secretion system includes the "core components" that form the apparatus in the wild type bacteria that injects factors into host cells or the surrounding medium, "effectors," which are transported by the core apparatus into the host cells or into the surrounding medium and "accessory factors" including chaperones and other components used by the wild type bacteria to control and effectuate the production and delivery of the effectors by the core apparatus.

Type III secretion system modifications in accordance with the present invention include any mutation that diminishes, abolishes or otherwise alters the effectiveness of the type III secretion system in performing any of the functions it carries *in vivo*. These modifications include, for example, mutations to the "core" proteins that decrease or abolish the ability of the system to secrete proteins or to translocate effectors into host cells or host cell membranes, mutations deleting or modifying effector genes, such that they are not produced or their function is attenuated, or mutations to other components of the system, chaperones for example, which are necessary for the delivery of the effectors in the wild type bacteria. Regulatory elements, transcription factors and other components used by the wild type bacteria may also be altered in such manner that the transcription, translation and/or processing of a component or components of the system is altered. *Bordetella* genetically engineered to include these mutations are also considered novel.

The generation of mutants is well known in the art. Loci coding for type III secretion system proteins or polypeptides may be altered in any effective manner. Preferred are deletions of the coding region for a particular protein or polypeptide, or the coding region of several proteins or polypeptides. Alternatively, an entire operon coding for such proteins and/or polypeptides may be deleted. Also preferred are in frame deletion or deletions of one or more codons of a protein or polypeptide, such that the function of the protein or polypeptide is abolished or attenuated. Also preferred is the substitution of one or more codons in one (or more) protein(s) or polypeptide(s) such that the function of the protein(s)

or polypeptide(s) is abolished or altered. Also preferred are insertional mutations. Combinations thereof may also be used.

Another aspect of the present invention is the use of genetically modified *Bordetella,* preferably *B. bronchiseptica*, as an effective mucosal antigen-delivery system, and as the component of a vaccine. Delivery of antigens to mucosal surfaces by expressing the antigens in the bacteria of the present invention is effective for inducing mucosal immunity. Systemic immunity may also be provided. The resulting immunity serves to protect the host from microbial, and the like, invasion, or to combat such organisms when present in the host.

When used as an antigen delivery system, *Bordetella* are genetically modified such that they may express one or more heterologous antigens. Any *Bordetella* may be used as a live vector, however *Bordetella* attenuated in accordance with the present invention are preferred. Most preferred are recombinant attenuated *Bordetella bronchiseptica*. In accordance with the present invention, *Bordetella* for use as a vaccine component or antigen delivery system may be attenuated by modifications to components other than to the type III secretion system, which nonetheless decrease or abolish export of type III secretion factors. For example, the strain Frl^r described in Akerley, et al., *Ectopic expression of the flagellar regulon alters development of the Bordetella-host interaction*, Cell 80(4):611-20 (1995), was engineered such that the *frlAB* promoter was replaced with that of *fhaB*. The entire motility regulon and flagellar proteins were expressed in the Bvg^+ rather than the Bvg^- phase. This mutant has now been discovered to not secrete most, if not all, type III wild type secreted proteins. The use of these modified *Bordetella*, and the like, as antigen delivery systems and as vaccine components is also considered novel.

The bacteria may also be further modified such that they do not express other toxic factors, for example hemolysin/adenylate cyclase toxin, dermonecrotic toxin, and the like. They may also be modified such that their transmissibility is attenuated or abolished, for example by mutating genes necessary for the Bvg^- phase, including motility genes, the *frlAb* locus, which encodes two proteins that function together as a transcriptional activator that regulates the motility regulon, the urase gene, the alcaligin gene, and the like. Mutants that are Bvg^+ phase-locked may also be used. As a precaution, DNA repair and DNA recombination enzymes, mating factors and accessory proteins, sites for integration of heterologous DNA, for plasmids or phages, for example, may also be altered such that the risk of the

modified *Bordetella* exchanging or incorporating DNA from other organisms in the environment are decreased.

Bordetella may be modified in any suitable manner such that it expresses the heterologous antigen. The DNA encoding the heterologous antigen may be present extra-chromosomally, but is preferably integrated into the bacterial chromosome. The construct may include regulatory sequences, ribosome binding sequences, and other elements necessary for the proper expression of the antigen or antigens. The construction and manipulation of these expression vectors are well known to those of skill in the art.

When the DNA encoding the heterologous antigen is present extra-chromosomally, for example in a plasmid, a balanced lethal host-vector system strategy may be used. *See, e.g.*, Curtiss, et al., *Stabilization of Recombinant Avirulent Vaccine Strains in Vivo*, Res Microbiol 141:797-805 (1993) (incorporated herein by reference). This strategy is preferred when the preservation of the plasmid and the expression of the antigen for prolonged periods is desired.

The antigens may be expressed under the control of any effective promoter, and may include other regulatory sequences and sequences necessary for the appropriate translation of the gene product. For example, constitutive promoters may be used. The use of the promoter for the recA gene (Kuhl, et al., *Isolation and characterization of the recA gene of Bordetella pertussis*, Mol Microbiol 7:1165-72 (1990)) is preferred. Alternatively, antigens may be expressed under the control of inducible promoters, which are also well known in the art. Preferred is the use of the inducible Tac promoter (Walker, et al., *Construction of minitransposons for constitutive and inducible expression of pertussis toxin in bvg-negative Bordetella Bronchiseptica*, Inf & Immun 59:4238-48). Preferred is the expression of the antigen(s) under the control of the *bvgAS* locus, which controls the expression of virulence factors in wild type *Bordetella*. The use of the regulatory sequences used by wild type *Bordetella* for the expression of virulence factors is most preferred. The construct may also be inserted in a functional manner into an operon having any of the above described characteristics.

Any antigen may be expressed, including multiple antigens. The antigen(s) may be expressed such that it remains within the bacteria, or may have a leader sequence such that the antigen is secreted, a transmembrane sequence or sequences, such that the antigen is anchored to the bacterial cytoplasmic or outer membranes, or a sequence such that it is

injected via the type III secretion system into the host cells. Combinations may also be used, such that the same antigen is displayed in the membrane, secreted, and/or expressed within the bacteria. When multiple antigens are expressed, they may be processed differently.

The antigens may also be expressed as fusion proteins. *See, e.g.*, Staats, et al., *Mucosal immunity to infection with implications for vaccine development*, Curr. Opin. Immunol 6:572-583 (1994) and references cited therein. Any effective fusion protein may be used. For example, for secretion the antigen may be fused to a protein or a portion of a protein that is secreted. Preferred are fusions to filamentous hemagglutinin, hemolysin/adenylate cyclase toxin, and the like, and portions thereof. For integration into the membrane, the antigen may be fused to any membrane protein. Preferred is the use of pertactin. For secretion via the type III secretion system the antigen may be fused to a protein secreted via the type III secretion system, and in particular it is desirable that about the first 10-20 codons of the secreted protein be present. *See, e.g.*, Anderson, et al., Yersinia enterocolitica *type III secretion: an mRNA signal that couples translation and secretion of YopQ*, Mol Microbiol 31(4):1139-48 (1999). The antigen may also be fused to other antigens, or to proteins known to have an adjuvant, or other desirable effect.

A protein or polypeptide, including for example an antigen, may be expressed as a fusion to a type III secreted gene so that it may be targeted into the host cytoplasm or membrane, or the extracellular medium by translocation of the fusion product via the type III secretion system. The strain expressing this construct may also be attenuated by specific inactivation of other type III secreted products (but not the "core" secretion apparatus genes) so that the only protein targeted into the host cell by type III secretion system is that of the fusion construct.

Antigens derived from *Leptospira canicola*, *L. grippotyphosa*, *L. hardjo*, *L. icterohaemorrhagiae*, *L. pomona*, *L. interrogans*, *L. bratislava*, canine distemper virus, canine adenovirus type 2, canine parainfluenza virus, canine parvovirus, rabies, herpes viruses, HIV, SIV, *Erysipelothrix rhusiopathiae*, *Pasteurella*, *P. multocida*, *Ascaris*, *Oesophagostomum*, pseudorabies virus, porcine parvovirus, pathogenic *E. coli*, including *E. coli* having K88, K99, 987P, and/or F41 adherence factors, *Clostridium spp.*, including *Cl. perfringens*, and *Cl. perfringens* type C beta toxoid, *Salmonella*, *Vibrio*, *Mycoplasma*, *Actinobacillus pleuropneumoniae*, *Haemophilus*, rotavirus, transmissible gastroenteritis virus,

Streptococcus sobrinus, *S. mutans*, influenza, to name a few, may be used. Other antigens, and antigens from other pathogens, which may be used in accordance with the present invention are within the skill and knowledge in the art. *Bordetella* factors, including virulence factors, may also be expressed, preferably in a modified form which prevents their deleterious effects while permitting the elicitation of an immune response specific to those factors.

Antigens expressed may be in the form of whole heterologous proteins, or portions thereof, and may be modified such that their toxicity is decreased, their stability increased, or such that the protein or polypeptide may have other suitable or desirable modifications. The expression of polypeptides consisting of protective epitopes is preferred. The antigen may be derived from any pathogen, toxin, or other immunogenic material, including viruses, bacteria, eukaryotes, unicellular and multicellular parasites, and combinations thereof, or small molecules may be produced by the expression of the appropriate enzymes in the modified *Bordetella*. As most agents either infect mucosal surfaces directly or gain entry to the body via mucosae or mucosal lymphoid tissue, immunization that elicits mucosal antibodies is effective in preventing and combating these agents.

The bacteria may also be engineered to directly or indirectly produce inflammatory stimuli. Direct inflammatory stimuli may be produced, for example, by the expression and secretion of pro-inflammatory cytokines (for a review, see Svanborg, et al., *Cytokine responses during mucosal infections: role in disease pathogenesis and host defence*, Curr Opin Microbiol 2(1):99-105 (1999)), including TNF, IL-1, IL-2, and other interleukins, TGF-β, interferon (for example INF-γ), GM-CSF, and the like, NO inhibitors (*see, e.g.*, Thepen et al., *The role of alveolar macrophages in regulation of lung inflammation*, Ann N Y Acad Sci. 725:200-6 (1994)), and the like. Enzymes or other proteins or polypeptides that may advantageously augment the immune response may be also be expressed.

In general, the genetically modified *Bordetella* may be used as an antigen delivery system in any animal that will support the modified *Bordetella* for a time sufficient to elicit an immune response. These include, for example, humans, dogs, cats, pigs, cows, sheep mice, rats, guinea pigs, rabbits, skunks, opossums, raccoons, ferrets, foxes, hedgehogs, koala, bears, leopards, horses and other animals.

The live attenuated *Bordetella* and the bacterial vectors of the present invention may be administered by any effective route. They are preferably administered such that the

bacteria colonize the nasal mucosa. The trachea may also be colonized, albeit at times only temporarily. Preferred is direct administration to the nasal mucosa, by spraying or injecting the bacteria suspended in a suitable solution, for example. Suitable solutions include water, saline, and other solutions known in the art. Inhalation and oral administration are also preferred.

In accordance with the present invention, the live attenuated *Bordetella* may be administered in any effective manner. The nasal route of administration is preferred. The reduced quantity of proteolytic enzymes and reduced antigenic competition in the nasal mucosa compared to the peroral and intragastric route may also present other advantages of *Bordetella* as a live attenuated vector.

In accordance with the present invention, the genetically modified *Bordetella* may be used as a component of a vaccine.

Bordetella having type III secretion mutants in accordance with the present invention which do not secrete type III secretion factors are also useful in identifying proteins and cloning DNA sequences encoding secreted factors. Factors can be cloned by their presence in the supernatant of cultured wild type bacteria and their absence from the supernatant of cultured modified bacteria. For example, wild type and modified bacteria may be separately grown in Stainer-Scholte medium at 37°C. Culture supernatants may then be precipitated (with TCA, or the like) separated by SDS-PAGE, and stained (with Coomassie blue, or the like). Polypeptides present in the wild type but absent from the modified bacterial supernatant may then be electroblotted (onto PVDF paper, for example) and their amino-terminal sequences determined (by Edman degradation, or the like). A *Bordetella* genomic or cDNA library, or the like, may then be screened to obtain isolated DNA sequences encoding type III secretion secreted factors, or PCR may be used to amplify and isolate the sequence, which may then be subcloned.

Alternatively, species A (mice for example) may be immunized with supernatant from wild type bacteria, while species B (rabbits, for example) may be immunized with supernatant from modified bacteria. In an expression screen of *Bordetella*, filters may be blocked with the serum from species B, then hybridized with the serum of species A. The filters are then incubated with labeled antibody specific for species A antibodies. As the clones producing products secreted by both wild type and mutant bacteria will have been blocked by antibodies present in the serum from the animals immunized with the mutant

bacteria (species B), the antibodies present in the serum from species A will only bind clones producing products secreted by the type III secretion, which will not have been blocked. The clones may then be isolated and the DNA sequences encoding the secreted factor(s) subcloned into an appropriate vector. Alternatively, a genetic strategy may be used. For example, Tn*cya*A mutagenesis to detect genes encoding polypeptides translocated into host cells, and the use of screens relying on regulatory factors specific to Type III gene expression to find co-regulated genes. Other like strategies may be used with the type III secretion mutants to clone DNA sequences encoding polypeptides and proteins secreted by the type III secretion system.

Another aspect of the present invention is a *Bordetella* type III secretion effector or combination of effectors, useful for sequestration of *NF-κB* in the cytoplasm. The effector, or effectors are also useful for preventing signal transduction to progress such that *NF-κB* activation and nuclear localization does not occur. The effector or effectors are also useful in preventing *NF-κB* from activating gene transcription, including preventing *NF-κB* from activating expression of cytokine and/or anti-apoptotic genes.

Another aspect of the present invention includes a *Bordetella* type III secretion effector or combination of effectors, useful for *in vivo* decreasing inflammation and increase apoptosis of inflammatory cells. These components find utility as anti-inflammatory agents, particularly at mucosal sites. Their use is particularly effective in the airways and lungs.

Another aspect of the present invention includes a *Bordetella* type III secretion effector or combination of effectors, with an in vivo tyrosine phosphatase activity.

The type III secretion system is also useful for testing compounds for antimicrobial agents. Agents that inhibit type III secretion of wild type *Bordetella* are effective in preventing the inhibition of inflammation, thus permitting the immune system of the host organism to clear the *Bordetella* infection.

Also provided by the invention are vectors that comprise the nucleic acid sequences described above. Examples of suitable vectors include but are not limited to retroviruses such as Moloney murine leukemia virus (MoMuLV); papovaviruses such as JC, SV40, polyoma, adenoviruses, Epstein-BalT Virus (EBV); papilloma viruses, e.g. bovine papilloma virus type I (BPV); vaccinia and poliovirus and other human and animal viruses.

Adenoviruses have several properties that make them attractive as cloning vehicles (Bachettis et al.: *Transfer of gene for thymidine kinase-deficient human cells by purified*

herpes simplex viral DNA, PNAS USA 74:1590 (1977); Berkner, K.L., *Development of adenovirus vectors for expression of heterologous genes*, Biotechniques, 6:616 (1988); Ghosh-Choudhury G, et al., Human adenovirus cloning vectors based on infectious bacterial plasmids, Gene; 50:161 (1986); Hag-Ahmand Y, et al., *Development of a helper-independent human adenovirus vector and its use in the transfer of the herpes simplex virus thymidine kinase gene*, J Virol 57:257 (1986); Rosenfeld K et al., *Adenovirus-mediated transfer of a recombinant al-antitrypsin gene to the lung epithelium in vivo*, Science 252:431 (1991)).

For example, adenoviruses possess an intermediate sized genome that replicates in cellular nuclei; many serotypes are clinically innocuous; adenovirus genomes appear to be stable despite insertion of foreign genes; foreign genes appear to be maintained without loss or rearrangement; and adenoviruses can be used as high level transient expression vectors with an expression period up to 4 weeks to several months. Extensive biochemical and genetic studies suggest that it is possible to substitute up to 77.5 kb of heterologous sequences for native adenovirus sequences generating viable, conditional, helper-independent vectors (Kaufman R.J.; *Identification of the component necessary for adenovirus translational control and their utilization in cDNA expression vectors*, PNAS USA 82:689 (1985)).

Another vector is AAV. AAV is a small human parvovirus with a single stranded DNA genome of approximately 5 kb. This virus can be propagated as an integrated provirus in several human cell types. AAV vectors have several advantage for human gene therapy. For example, they are trophic for human cells but can also infect other mammalian cells; (2) no disease has been associated with AAV in humans or other animals; (3) integrated AAV genomes appear stable in their host cells; (4) there is no evidence that integration of AAV alters expression of host genes or promoters or promotes their rearrangement; (5) introduce genes can be rescued from the host cell by infection with a helper virus such as adenovirus.

HSV-1 vector system facilitates introduction of virtually any gene into non-mitotic cells (Geller et al., An efficient deletion mutant packaging system for a defective herpes simplex virus vectors: Potential applications to human gene therapy and neuronal physiology, PNAS USA 87:8950 (1990)).

Another vector for mammalian gene transfer is the bovine papilloma virus-based vector (Sarver N, et al., *Bovine papilloma virus DNA: A novel eukaryotic cloning vector*, Mol Cell Biol 1:486 (1981)).

Vaccinia and other poxvirus-based vectors provide a mammalian gene transfer system. Vaccinia virus is a large double-stranded DNA virus of 120 kilodaltons (kd) genomic size (Panicali D, et al., *Construction of poxvirus as cloning vectors: Insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccine virus*, Proc Nad Acad Sci USA 79:4927 (1982); Smith et al., *Infectious vaccinia virus recombinants that express hepatitis B virus surface antigens*, Nature 302:490 (1983).)

Retroviruses are packages designed to insert viral genes into host cells (Guild B, et al., *Development of retrovirus vectors useful for expressing genes in cultured murine embryonic cells and hematopoietic cells in vivo*, J Virol 62:795 (1988); Hock RA, et al., *Retrovirus mediated transfer and expression of drug resistance genes in human hemopoietic progenitor cells*, Nature 320:275 (1986)).

The basic retrovirus consists of two identical strands of RNA packaged in a proviral protein. The core surrounded by a protective coat called the envelope, which is derived from the membrane of the previous host but modified with glycoproteins contributed by the virus.

Further, microorganisms are provided that are transformed by vectors including DNA sequences encoding constituents of the secretory system of the present invention. In accordance with the practice of the invention, the microorganism is *Bordetella*, *e.g.*, *Bordetella* selected from the group including, but not limited to, *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. The invention further provides host cells infected with the microorganisms of the invention.

Additionally, vaccines for protecting an animal against a disease are provided. For example, the vaccine can induce a cytotoxic T lymphocyte (CTL) mediated immune response. In one embodiment of the invention, the vaccine comprises a sufficient amount of microorganisms of the invention. The microorganisms of the invention have been transformed by the vectors of the invention. In accordance with the practice of the invention, a vaccine can further comprise a sufficient amount of one or more additional antigenic components for protecting an animal against disease caused by one or more other pathogenic microorganism, cells or viruses. Examples of suitable antigenic components include but are not limited to one or more of inactivated leptospira canicola, inactivated

leptospire icterhemmorhagiae, modified canine distemper virus, modified canine adenovirus type 2, modified canine parainfluenza virus and modified canine parvovirus.

The invention further provides hybrid nucleic acid sequences. These hybrid nucleic acid sequences encode heterologous gene products and comprise nucleic acid sequences encoding members of a type III secretion system for *Bordetella* joined to a transgene or multiple transgenes. These heterologous gene products can stimulate host immunity, e.g., by a CTL mediated immune response, and serve as a vaccine for *Bordetella* or non-*Bordetella* infection. In one embodiment, the transgene encodes an immunogenic protein from any species. Examples of suitable immunogenic proteins include but are not limited to a pilinic subunit, pertussis toxin or subunits thereof, filamentous hemagglutinin, adenylate cyclase and the protein 69K.

In one embodiment, the hybrid nucleic acid sequence includes, however is not necessarily limited to bscV, bcr3, bopN, bsp22, bcrH1, bopD, bopB, bcrH2, bcr4, bscl, bscJ, bscK, bscL, bscN and bscO having the sequences described above and a transgene.

Examples of transgenes include suicide genes and genes that show late cell development. For example, suicide result in a protein or agent that inhibits cell growth or tumor cell death. Suicide genes include genes encoding enzymes, oncogenes, tumor suppressor genes, genes encoding toxins, genes encoding cytokines, or a gene encoding oncostatin. The purpose of the transgene is to inhibit the growth of or kill cells, of interest or produce cytokines or other cytotoxic agents which directly or indirectly inhibit the growth of or kill the cell of interest.

Suitable enzymes include thymidine kinase (TK), xanthine-guanine phospho-ribosyltransferase (GPT) gene from *E. coli* or *E. coli* cytosine deaminase (CD), or hypoxanthine phosphoribosyl transferase (HPRT).

Suitable oncogenes and tumor suppressor genes include neu, EGF, ras (including H, K, and N ras), p53, Retinoblastoma tumor suppressor gene (Rb), Wilm's Tumor Gene Product, Phosphotyrosine Phosphatase (PTPase),, and nm23. Suitable toxins include *Pseudomonas* exotoxin A and S; diphtheria toxin (DT); *E. coli* LT toxins, Shiga toxin, Shiga-like toxins (SLT-1, -2), ricin, abrin, supporin, and gelonin.

Suitable cytokines include interferons, GM-CSF interleukins, tumor necrosis factor (TNF) (Wong G, et al., *Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins*, Science 228:810 (1985));

W09323034 (1993); Horisberger MA, et al., *Cloning and sequence analyses of cDNAs for interferon- and virus-induced human Mx proteins reveal that they contain putative guanine nucleotide-binding sites: functional study of the corresponding gene promoter*, Journal of Virolo 64(3):1171-81 (1990); Li YP et al., Proinflammatory cytokines tumor necrosis factor-alpha and IL-6, but not IL-1, down-regulate the osteocalcin gene promoter, Journal of Immunology 148(3):788-94 (1992); Pizarro TT, et al., *Induction of TNF alpha and TNF beta gene expression in rat cardiac transplants during allograft rejection*, Transplantation 56(2):399-404 (1993)). Breviario F, et al., *Interleukin-1-inducible genes in endothelial cells. Cloning of a new gene related to C-reactive protein and serum amyloid P component.* Journal of Biologic Chemistry, 267(31):22190-7 (1992); Espinoza-Delgado I, et al., *Regulation of IL-2 receptor subunit genes in human monocytes. Differential effects of IL-2 and FFN-gamma*, Journal of Immunology 149(9):2961-8 (1992); Algate PA, et al., *Regulation of the interleukin-3 (IL-3) receptor by IL-3 in the fetal liver-derived FL5.12 cell line*, Blood 83(9)-2459-68 (1994); Cluitmans FH, et al., *IL-4 down-regulates IL-2-, IL-3-, and GM-CSF-induced cytokine gene expression in peripheral blood monocytes*, Annals of Hematology, 68(6):293-8 (1994); Lagoo, AS, et al., *IL-2, IL-4, and IFN-gamma gene expression versus secretion in superantigen-activated T cells. Distinct requirement for costimulatory signals through adhesion molecules*, Journal of Immunology 152(4):1641-52 (1994); Martinez OK et al., *IL-2 and IL-5 gene expression in response to alloantigen in liver allograft recipients and in vitro*, Transplantation 55(5):1159-66 (1993); Pang G, et al., *GM-CSF, IL-1 alpha, IL-1 beta, IL-6, IL-8, IL-10, ICAM-1 and VCAM-1 gene expression and cytokine production in human duodenal fibroblasts stimulated with lipopolysaccharide, IL-1 alpha and TNF-alpha*, Clinical and Experimental Immunology 96(3):437-43 (1994); Ulich TR, et al., *Endotoxin-induced cytokine gene expression in vivo. III. IL-6 mRNA and serum protein expression and the in vivo hematologic effects of IL-6*, Journal of Immunology 146(7):2316-23 (1991); Mauviel A, et al., *Leukoregulin, a T cell-derived cytokine, induces IL-8 gene expression and secretion in human skin fibroblasts. Demonstration and secretion in human skin fibroblasts. Demonstration of enhanced NF-kappa B binding and NF-kappa B-driven promoter activity.*, Journal of Immunology 149(9):2969-76 (1992)).

Growth factors include Transforming Growth Factor-α (TGFα) and β (TGFβ), cytokine colony stimulating factors (Shimane M, et al., *Molecular cloning and characterization*

of G-CSF induced gene cDNA, Biochemical and Biophysical Research Communications 199(1):26-32 (1994); Kay AB, et al., *Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects*, Journal of Experimental Medicine 173(3):775-8 (1991); de Wit H, et al., *Differential regulation of M-CSF and IL-6 gene expression in monocytic cells*, British Journal of Haematology 86(2):259-64 (1994); Sprecher E, et al., Detection of IL- I beta, TNF-alpha, and IL-6 gene transcription by the polymerase chain reaction in keratinocytes, Langerhans cells and peritoneal exudate cells during infection with herpes simplex virus-1, Archives of Virology 126(1-4):253-69 (1992)).

Preferred vectors for use in the methods of the present invention are viral including adenoviruses, retroviral, vectors, adeno-associated viral (AAV) vectors. However, other vectors generally known in the art may be used.

The invention also provides recombinant toxin antigens encoded by any of the hybrid nucleic acid sequences described above. Further, the invention provides vaccine compositions comprising pharmaceutically acceptable carriers or diluents in combination with any of the recombinant toxin antigens of the invention. In one embodiment, the vaccine composition further comprises an antigen adjuvant.

The invention additionally provides antibodies or derivatives thereof which recognize and bind a protein of the type III secretion system of the invention derived from type III secretion system of *Bordetella*.

The invention provides methods for protecting an animal (e.g., a dog, cat, pig, or cow) against disease. In one embodiment a method which comprises administering to the animal a vaccine. The vaccine comprises a microorganism of the invention in an amount sufficient to protect the animal. Administration of the vaccine can be effected by parenteral injection, intranasal administration, intrapharyngeal administration, or topical administration. Other means of administration are possible.

In accordance with the practice of the invention, the animal can be a dog and protection is directed to prevention of kennel cough. In this case, administration of the vaccine can be effected by intrapharyngeal application. Other administration means are possible.

Alternatively, the animal is a swine and protection is directed to prevention of atrophic rhinitis and turbinate atrophy. In this case, administration can be effected by intranasal application. Other administration means are possible.

In accordance with the practice of the method of the invention, the amount of the microorganism to be administered is at least one microorganism per administration. The amount of the microorganism can be in a range of 1 microorganism to 100 million microorganisms per administration.

The invention also provides a method for protecting an animal against disease which comprises administering to the animal a vaccine composition comprising a pharmaceutically acceptable carrier or diluent in combination with any of the recombinant toxin antigen of the invention.

Additionally, the invention provides methods for expressing a heterologous gene product from a *Bordetella* strain. The method comprises attaching a transgene to a nucleic acid sequence of the invention so as to produce a hybrid gene. Further, the method provides introducing the hybrid gene into the *Bordetella* strain to form a viable transformed *Bordetella* strain and culturing the transformed *Bordetella* strain to effect expression of the heterologous gene product. In one embodiment, expression of the heterologous gene product is constitutive, or regulated.

The invention also provides methods for the diagnosis of diseases associated with a *Bordetella* strain in a subject. This method comprises obtaining a specimen, *e.g.*, of nasopharyngeal secretions, from the subject and detecting the presence of a nucleic acid sequence of the invention. After detection is effected, the method provides quantitatively determining the number of nucleic acid sequences so present. In accordance with the practice of the invention quantitatively determining the number of nucleic acid sequences can be achieved by comparing the number of cells so detected to the amount in a sample from a normal subject. The presence of a measurable different amount indicating the presence of the disease.

Alternatively, the number of nucleic acid sequences can be achieved by comparing the number of cells so detected to the amount in a sample from the same subject at a different point in time so that a difference can be determined. The difference in time being indicative of the state of the disease.

In accordance with the practice of the invention the subject includes, but is not limited to, a dog, cat, pig, cow.

The invention further provides methods for detecting the presence of nucleic acid sequences encoding a proteins of the type III secretion system for *Bordetella* in a sample.

In one embodiment, the invention comprises contacting a protein encoded by a nucleic acid with the antibody of the invention thereby forming a detectable complex. The presence of the complex in the sample is indicative of the presence of the nucleic acid sequence. In accordance with the practice of the invention, the antibody can be labeled so as to directly or indirectly produce a detectable signal. The label includes but is not limited to a compound such as a radiolabel, an enzyme, a chromophore and a fluorescer.

Further, in another embodiment, the invention comprises contacting the sample with any of the nucleic acid sequence of the invention and detecting the binding of the nucleic acid to a constituent in the sample thereby forming a complex. The presence of the complex being indicative of the nucleic acid encoding any of the bscV, bcr3, bopN, bsp22, bcrH1, bopD, bopB, bcrH2, bcr4, bscl, bscJ, bscK, bscL, bscN and bscO protein in the sample. In accordance with the practice of the invention, the constituent can be a DNA or RNA. Further, the sample can be a tissue or biological fluid sample. Examples of biological fluids include but is not limited to urine and blood sera.

In accordance with the practice of the invention, the nucleic acid sequence can be labeled so as to directly or indirectly produce a detectable signal. Typically, the label is a compound selected from the group consisting of a radiolabel, an enzyme, a chromophore and a fluorescer. Other compounds are possible.

The invention further provides a method for producing a *Bordetella* bacteria having an inactivated secretion system. This method comprises genetically modifying the *Bordetella* bacteria by removing any of the type III secretion genes, e.g., any or all of the nucleic acid sequences of invention. Alternatively, the method comprises genetically modifying the *Bordetella* bacteria by modifying any of the type III secretion genes so as to inhibit secretion or transfer of the gene product to the host. The genetically modified *Bordetella* having an inactivated secretion system so produced can be used as an attenuated strains for a vaccine against a *Bordetella* infection.

Further, the invention provides a method for inhibiting infectious *Bordetella* bacteria in a subject by administering the genetically modified *Bordetella* so produced by the

method above to a subject. Transient or limited colonization by the modified Bordetella results in protection against infectious bacteria.

The type III system of the invention can be used to deliver macromolecules, *e.g.*, proteins of interest, directly into host cells. There has been no previous report of a type III secretion system in *Bordetella spp.* and all of the previously identified protein toxins synthesized by *Bordetella spp.* do not appear to be secreted by a type III system. The data herein shows that genes of a type III secretion apparatus when expressed, *e.g.*, in *B. bronchiseptica,* is regulated by the BvgAS two-component signal transduction system.

The regulation of type III secretion is useful since secretion of many proteins via this pathway appears to be facilitated by direct contact between bacteria and target cells (Galan and Bliska, 1996). Environmental signals including pH, redox potential, osmolarity, and nutrient levels can also regulate expression of type III system genes (Bajaj et al., 1996; Lee, 1997).

The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.

EXAMPLES

Experimental procedures

Bacterial strains and growth conditions.

B. bronchiseptica strains RB50, RB54 and RB53; *B. pertussis* strains Tohama 1, 18323 and GMT1; ovine isolate of *B. parapertussis* strain H1 have all been previously described (Cotter and Miller, 1994; Martinez de Tejada et al., 1996; Porter et al., 1994). *B. pertussis* strain 17474 was a clinical isolate from Erlangen, Germany and human isolate of *B. parapertussis* strain A168 was from CDC, USA. *B. bronchiseptica* strain FF1 was constructed from RB54 by replacing wild type promoters of *fhaB* and *fhaC* with the *flaA* promoter to ectopically express FHA. All strains were cultured in Stainer-Scholte liquid medium or BG agar as previously described (Cotter and Miller, 1994; Cotter and Miller, 1997; Martinez de Tejada et al., 1996).

Differential display, PCR, RT-PCR, RNA slot blots, DNA sequencing and molecular cloning.

Total RNA was isolated from mid-log bacterial cultures using Trizol reagent (Gibco) according to the manufacturer's protocol. Total RNA was reversed-transcribed into cDNA

using 2μg RNA, 200 ng random hexamers and Superscript II (Gibco) as described by manufacturer's protocol. Reaction conditions for differential display/arbitrary-primed PCR were as follows: One-tenth or one-twentieth of the cDNA from each reverse-transcription reaction was combined with 3mM $MgCl_2$, 1 U Taq Polymerase (Promega, Madison WI), 250 μM each of the 4 dNTPs and 20 pmoles of primer in a total volume of 25 μl. A PTC-100 thermal cycler (MJ Research) was used for the reactions. For short primers (10-mers), the cycling parameters were: 45 cycles of (94°C for 1 min, 36°C for 1 min, 72°C for 2 min) and then 72°C for 5 min. For longer primers (>17 mers): 4 cycles of (94°C for 5 min, 40°C for 5 min and 72°C for 5 min) were followed by 30 cycles of (94°C for 1 min, 55°C for 1 min and 72°C for 2 min) and a final incubation at 72°C for 5 min.

PCR products were analyzed on 2% agarose gels containing 0.5 μg/ml ethidium bromide. To determine reproducibility, reactions with different concentrations of cDNA were run in duplicates. Specific bands of interest were isolated with Geneclean kit (Biol0l), reamplified under the same PCR conditions, and then cloned using the TA cloning kit (Invitrogen).

For PCR with specific primer pairs, conditions were as follows: 2 mM $MgCl_2$, 1 U Taq Polymerase (Promega, Madison WI), 200 μM each of the 4 dNTPs, 1 μM of each primer and 5% DMSO. For genomic DNA templates, a single colony of bacterial culture on a plate was picked and directly mixed into the reaction solution. For cDNA templates, one-twentieth of each reverse-transcription reaction was used. Cycling parameters were: 25 cycles of (94°C for 1 min, 55° or 50°C for 1 min and 72°C for 1 min) and a final incubation at 72°C for 5 min. Sequence of primers used are shown in Table 2.

RNA slot blots were performed as previously described (Cotter and Miller, 1997). Probes used were: a 500 bp EcoRI-StyI fragment of *recA*, a 450 bp *BglII-BamHI* fragment of *fhaB*, a 500 bp PCR product of *fla*A using primers BA04 and BA01 1, and a 420 bp PCR product of *bscN* using primers W3 and W4. All recombinant DNA techniques were performed as described in standard protocols (Sambrook et al., 1989). *Bacterial conjugations, allelic exchanges, plasmid rescues and construction of in-frame deletion.*

Allelic exchanges were performed using suicide vectors pEG7 or pEGBR (Akerley et al., 1995; Cotter and Miller, 1997; Martinez de Tejada et al., 1996). DNA fragments used for homologous recombinations were subcloned into the vectors and then transformed into *E. coli* SM10 for mating to *B. bronchiseptica*. Matings, selection for gentamicin or

kanamycin resistant co-integrants and counter-selection against sucrose sensitivity for second recombination events were done as described (Akerley et al., 1995; Cotter and Miller, 1997; Martinez de Tejada et al., 1996).

DNA flanking original fragment of *bscN* (from arbitrary-primed PCR) was isolated as follows: the 420 bp PCR fragment was subcloned into pEG7 and the resulting suicide plasmid introduced into RB50. Genomic DNA from gentamicin resistant colonies (containing integrated plasmid by homologous recombination into the *bscN* gene) was digested with *NsiI* (one of several restriction enzymes used which does not cut within pEG7), self-ligated, transformed into E. coli XL1-Blue and selected by ampicillin resistance. The rescued plasmid containing extra 4 kb of DNA was restriction mapped and fragments were subcloned into pBluescript for DNA sequencing on both strands. The assembled sequence was analyzed for ORFs and searched for homologous sequences in the database using BLAST (NCBI) and sequence alignments were performed with ALIGN in the FASTA program (U. Virginia).

For construction of the in-frame deletion in *bscN*, two PCR fragments using primers W1+W2, which amplify a 350 bp fragment (from codon no. 54 to codon no. 170 of the *bscN* ORF) and primers W3+W4, which amplify a 420 bp fragment (from codon 262 to 400) were ligated by overlapping PCR, using overlapping regions between W2 and W3, in the presence of primers W1 and W4. Pfu polymerase (Stratagene) was used for these PCRs. The resultant 770 bp fragment was sequenced to ensure maintenance of the reading frame and then subcloned into pEGBR. The resulting suicide vector was introduced into RB50 and two recombination events were selected for (first by kanamycin resistance and then by sucrose resistance). Resulting colonies were screened by PCR with primers W1 and W4 which give a 770 bp product from the genome of the deletion strain WD3 but a 1050 bp product from the wild type. For construction of the transcriptional *LacZ* fusion with *bscN*, the 420 bp PCR product from W3+W4 was subcloned into the suicide vector pEGZ (Martinez de Tejada et al., 1996), integrated into RB50 genome by homologous recombination, and selected by gentamicin resistance.

For construction of the in-frame deletion in *bsp22*, two PCR fragments using primers

D21 (5'GCGGATCCAGTTTTGCCTGCGCGTCG3') and

D22 (5'AACTCCGAGATCAATGGTCATG3')

which amplify a 490 bp fragment (from upstream of *bsp22* ORF to including first 7 codons) and primers

D23 (5'ATGACCATTGATCTCGGAGTTAACAGTTCCATCACCAACAAC3') and

D24 (5'GCGGATCCAACCCCTGCAAGCTGCCC3')

which amplify a 460 bp fragment (from the last 9 codons to downstream of *bsp22* ORF) were ligated by overlapping PCR, using overlapping regions between D22 and D23, in the presence of primers D21 and D24. The resultant 930 bp fragment was sequenced to ensure maintenance of the reading frame and then subcloned into pEGBR. The resulting suicide vector was introduced into RB50 (via conjugation with transformed *E. coli* SM10 strains) and two recombination events were selected for (first by kanamycin resistance and then by sucrose resistance). Resulting colonies were screened by PCR with primers D21 and D24 that gave a 930 bp product from the genome of the deletion strain D218 but a 1490 bp product from the wild type. Functional deletion of Bsp22 in D218 was confirmed by immunoblots. For complementation of D218, an 850 bp fragment consisting of the entire ORF of *bsp22* was obtained by PCR with the primers

LB25K (5'ACTGGTACCTCGGAGAAGGAACCATTTGCCTAC3') and

B2FF3B (5'CTAGGATCCGCGGCACGCATGGATTGG3')

which was subcloned into KpnI and BamHI sites of the broad host range plasmid pBBR1MCS (Kovach, et al., *Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes*, Gene 166(1):175-6 (1995)) to create the plasmid pLB2. pLB2 was transformed into *E. coli* stain SM10 which in turn was conjugated to D218 and resultant ex-conjugants selected by antibiotic resistance.

SDS-PAGE, Western immunoblots and ELISA, protein sequencing, antibody production and β-galaclosidase assays.

Protein analysis on SDS-PAGE and immunoblots were performed as previously described (Cotter and Miller, 1997; Martinez de Tejada et al., 1996). Proteins from 15 hour culture supernatants were first precipitated with 10% trichloroacetic acid (TCA) for 4 hours on ice. 10 ml equivalent of supernatant was used for each lane loaded for detection by Coomassie staining while 1 ml equivalents were used for immunoblots. Total cellular proteins from L2 cell cultures were obtained by solubilizing cells on 6-well tissue culture plates with 300 µl of protein sample buffer and then scraped off and boiled for 5 minutes. One-tenth of that was loaded on each lane. Sera from rabbits, rats and mice previously

infected with *B. bronchiseptica* for 1 to 2 months were diluted 1:2500 for immunoblotting. Anti-phosphotyrosine monoclonal antibody from mouse was clone PT-66 from Sigma and it was used at 1:2000 dilution for immunoblots. All detections of immunoblots were done with enhanced chemiluminescence (Amersham). P-galactosidase assays were performed as previously described (Martinez de Tejada et al., 1996).

For protein amino-terminal sequence determination, proteins separated by SDS-PAGE were electroblotted onto PVDF paper and stained with Coomassie Blue. A GST fusion of the entire ORF of bsp22 was constructed and purified with standard protocols using pGEX2T vector (Pharmacia). 8 week old BALB/c mice were injected i.p. with 100 μg of the GST-fusion protein in a 1:9 emulsion with complete Freund's adjuvant on days 0, 14, 28, 35 and 42 to induce antibody and ascites production. Ascites from the mice were collected on days 35, 42 and 49, and tested for specificity to antigens with immunoblots. Rabbit and mouse antisera were diluted 1:2500 for immunoblotting. All detections of immunoblots were done with enhanced chemiluminescence (Amersham).

Mammalian cell cultures, cytotoxicity assays, apoptosis assays and immunofluorescent studies.

L2 rat lung epithelial cell line (ATCC) was cultured in F12K medium supplemented with 10% fetal bovine serum (FBS) while J774 and RAW macrophage-like cell lines were cultured in DME with 10% FBS. L2 cells were grown to 70% confluency for cytotoxicity assays and 95% confluency for phosphotyrosine assay. Bacterial infections of the cell cultures were done with multiplicity of infection (MOI) ranging from 10 (for macrophage cell lines) to 500 (for L2 cells) and bacterial suspensions were centrifuged onto the adherent cells at 500Xg for all the assays. For L2 cytotoxicity assays, at the end of incubations, the cells were washed three times with Hanks Balanced Salt Solution (HBSS), fixed in methanol and stained with Giemsa stain for 30 minutes before observations under a phase-contrast microscope. Cytotoxicity assays from the macrophage cell lines were performed using the Cytotox96 kit (Promega) as per manufacturer's protocol.

Apoptosis assays were performed using the *in situ* cytotoxicity assay kit based on a fluorescent TUNEL assay (Boehringer Mannheim) according to manufacturer's protocol. Cytotoxicity assays for the J774A.1 cell line were performed using the Cytotox96 kit (Promega) according to manufacturer's protocol. Immunofluorescent labeling of L2 cells were performed as follows: cells were fixed for 30 minutes in 4% paraformaldehyde and

permeabilized with 0.1% TritonX-100 in PBS for 10 minutes. Fixed samples were pre-incubated for 30 minutes in 1% BSA/Hanks balanced salt solution (HBSS) with 1:100 dilution of normal goat serum, followed by 60 minutes in 1:200 dilution of primary antibody (against p65 subunit of NF-κB, sc-372, Santa Cruz Biotechnology) in the same buffer. They were then washed 3 times in 1% BSA/HBSS and then incubated with 1:200 dilution of Alexa568 tagged goat anti-rabbit antibody (Molecular Probes) for 30 minutes. Cells were then washed 3 times in PBS and observed under epifluorescent microscopy.

Experimental animals.

Infection of 4 week old female Wistar rats with either RB50 or WD3 was done as previously described (Akerley et al., 1995; Cotter and Miller, 1997; Martinez de Tejada et al., 1996). 1000 cfu of each strain in a 5 μl volume was inoculated intra-nasally. Statistical significance of differences in colonization was determined by unpaired t-test comparisons. Statistical significance of the differences in survival time was determined by Logrank (Mantel-Cox) comparisons. All experiments were in accordance with guidelines and protocols approved by UCLA Animal Research Committee.

Identification and cloning of a yscN *homologue from* B. bronchiseptica.

The technique of DD-PCR was used with arbitrary primers to identify genes that are differentially expressed under Bvg^+ or Bvg^- growth conditions in *B. bronchiseptica in vitro*. Total RNA was isolated from *B. bronchiseptica* strain RB50 grown under non-modulating (Bvg^+) or modulating (Bvg^-) conditions and reverse-transcribed to cDNA. The cDNAs were used as templates for PCR amplification using arbitrary primers ranging from 10 to 35 nucleotides in length. Several primers amplified distinct fragments that were specific to the Bvg^+ phase. One of the primers, KB4, produced a 480 bp band from Bvg^+ phase cDNA only (Fig. 1, lanes 1 and 2). This band was cloned, sequenced and found to correspond exactly to an internal sequence of *cyaA*, the Bvg-activated gene encoding adenylate cyclase toxin/hemolysin. Another primer, ML2, produced a 420 bp fragment specifically from Bvg^+ phase, but not Bvg^- phase cDNA (Fig. 1, lanes 5 to 8). The DNA and the predicted amino acid sequence of this fragment showed a high degree of similarity to the 3' end of the ORF for the *yscN* gene from *Yersinia spp.* (Bergman et al., 1994, Woestyn et al., 1994). The YscN gene product is postulated to hydrolyze ATP to provide energy for secretion of Yops (*Yersinia* outer proteins) via the type III secretion system. There has been no previous report of type III secretion genes in *Bordetella spp.* The 420 bp RT-PCR fragment was used

to clone the complete ORF and flanking genes by plasmid rescue (described in Experimental procedures). The 420 bp fragment lies within an ORF encoding a predicted 48 kD protein with a 64% amino acid identity to YscN from *Yersinia* (Fig. 2a). This *B. bronchiseptica* protein was designated BscN.

BscN contains two conserved Walker Boxes (Walker et al., 1982), suggesting it can bind and hydrolyze ATP. Fourteen other ORFs flank *bscN* (Fig. 3). Thirteen of these ORFs show amino acid sequence similarities to proteins in *Yersinia spp.* which comprise part of the Ysc type III secretion apparatus (Allaoui et al., 1995; Bergman et al., 1994), and also the corresponding Psc homologues in *Pseudomonas aeruginosa* (Yahr et al., 1996). The homologous ORFs were designated in *B. bronchiseptica* as bscV, bcr3, bopN, bcrH1, bopD, bopB, bcrH2, bcr4, bscl, bscJ, bscK, bscL, bscN and bscO. The nucleotide sequence of a 4.6 kb fragment containing some of these genes have been deposited in Genbank under the accession number AF049488 (incorporated herein by reference in their entirety). A 13500bp sequence corresponding to the 15 ORF and flanking sequences is presented in figure 18. The organization of these ORFs suggests that they may be transcribed as an operon.

BscN *expression is positively regulated by BvgAS.*

Three assays were used to investigate the role of BvgAS in the control of *bscN* expression: RT-PCR, slot blot hybridizations of total RNA and *lacZ* transcriptional fusion analysis. Figure 4a shows results of RT-PCR using various primer pairs on genomic or cDNA templates prepared from RB50 grown under Bvg^+ or Bvg^- phase conditions. Primers specific for the filamentous hemagglutinin (FHA) structural gene (*fhaB*), a Bvg^+ phase factor, gave rise to a PCR product of the correct size only from cDNA made from Bvg^+ phase RNA. Primers specific for flagellin (*fhaA*), a Bvg^- phase gene, gave rise to a PCR product only from cDNA made from Bvg^- phase RNA. These controls confirmed that the RNAs from Bvg^+ and Bvg^- phase grown RB50 contain phase-specific transcripts. Mock reactions, which did not contain reverse transcriptase in the RT reactions, did not give rise to PCR products, confirming negligible genomic DNA contamination in the RNA preparations. The two pairs of primers specific for bscN (W1+W2, which amplify a 350 bp region in the 5' end; and W3+W4, which amplify a 420 bp region in the 3' end) generated products of the expected sizes only from cDNAs derived from Bvg^+ phase RNA. cDNAs were also prepared from Bvg^+ and Bvg^- phase-locked derivatives of RB50, RB53 and

RB54, respectively. Only cDNAs prepared from RB53 RNA generated PCR products of the expected sizes with primers specific for *bscN* (Fig. 4b). RT-PCR with primers specific for the *bscIJKL* loci produced the same results, showing that they were transcribed only in the Bvg^+ phase.

To confirm the results from RT-PCR, a DNA probe specific for 420 bp of the 3' end of *bscN* was used to hybridize to total RNA on slot blots (Fig. 4c). The control probes for transcripts of *fhaB* (a Bvg^+ phase locus), *flaA* (a Bvg^- phase locus) and *recA* (not regulated by Bvg) showed that the RNA preparations were quantitatively loaded and Bvg phase specific. The probe for *bscN* showed a much higher degree of hybridization to the RNA derived from Bvg^+ phase compared to Bvg^- phase grown cultures. Finally, a strain of RB50 containing a transcriptional fusion of the *lacZ* gene integrated into the 3' end of the chromosomal *bscN* locus was constructed. Beta-galactosidase assays of this strain grown under Bvg^+ and Bvg^- phase conditions during exponential growth showed a twenty-five-fold induction in Bvg^+ phase (β-galactosidase units of 362±65 in Bvg^+ phase versus 13±5 units in the Bvg^- phase). *bscN* expression is tightly regulated by the *bvg* locus and it is transcribed preferentially in the Bvg^+ phase.

BscN is required for secretion of specific Bvg^+ phase polypeptides.

Absence of a functional *bscN* abolishes type III secretion. An in-frame deletion in the RB50 *bscN* locus was constructed as described above. The resulting strain, designated WD3, has a deletion that removes both Walker Boxes (see Fig. 2a) and should render the BscN protein functionally inactive, based on data from similar mutations in *Yersinia spp.* (Woestyn et al., 1994). WD3 shows a similar growth rate in Stainer-Scholte medium compared to that of RB50 (doubling time of 85 minutes for WD3 versus 90 minutes for RB50 at 37°C with aeration).

To determine if BscN is involved in protein secretion in *B. bronchiseptica*, the protein profile of culture supernatants from RB50 and WD3 was compared. WD3 produced and secreted FHA at levels comparable to RB50 based on immunoblots and also showed the same degree of hemolysis on BG blood agar, suggesting the secretion of FHA and AC/HLY were not noticeably affected by the *bscN* mutation. Total cellular protein preparations from RB50 and WD3 showed very similar profiles on SDS-PAGE based on Coomassie staining and on immunoblots with post-infection sera against *B. bronchiseptica*. However, comparison of Coomassie stained proteins from supernatants of 15 hour cultures of RB50

and WD3 grown under Bvg^+ phase conditions revealed several polypeptides in the culture supernatants from RB50 but not WD3 (Fig. 5, lane 1 and 2, open arrows).

Immunoblot analysis of concentrated supernatant with sera from a rabbit, rat and mouse previously infected with RB50 also identified proteins secreted by RB50 but not by WD3 (Fig. 5, lanes 3 to 9, solid arrows). These proteins are secreted only in the Bvg^+ phase as none of them were detected in the culture supernatant of a Bvg^- phase-locked strain, RB54 (lane 5). Interestingly, the most prominent bands observed on immunoblots did not correspond to the most significant bands detected by the Coomassie staining, with the exception of the 22kD band. Although the antisera were from different mammalian species, they all recognized the same proteins that were differentially secreted from RB50 and WD3. When antisera from animals infected with WD3 were used as probes, none of the differentially secreted proteins were detected (Fig. 5, lanes 10 to 13). This correlates with the observation that the proteins were secreted from RB50 and not WD3. These results indicate that the in-frame deletion in *bscN* leads to a significant decrease of a subset of Bvg^+ phase polypeptides in the culture supernatants. Detection of these bscN-dependent secreted proteins by antisera from RB50 infected animals indicates that they are antigenic and expressed *in vivo*.

At least 9 polypeptides are secreted by type III secretion apparatus in *B. bronchiseptica*.

In-frame deletion of bscN *leads to decreased cytotoxicity of* B. bronchiseptica *to mammalian cells* in vitro.

B. bronchiseptica adheres to a variety of mammalian cells in culture including L2 rat lung epithelial cells and J774 macrophage-like cells. In both cases, binding is Bvg^+ phase dependent. After prolonged binding of RB50 to L2 cells (2 hours or more), it was observed that the L2 cells began to show signs of toxicity: the cytoplasm shrinks, cells round up and detach from the culture plates (Fig. 6c). The *bscN* deletion strain WD3, which in the Bvg^+ phase bound to L2 cells with similar efficiency as RB50, did not lead to the same degree of observable toxicity to L2 cells over the same time period (Fig. 6f). A Bvg^- phased-lock strain which has been engineered to ectopically express FHA (strain FF1) but does not express other Bvg^+ phase factors, could bind to L2 cells but did not elicit any signs of cytotoxicity for over 2 hours (Fig. 6d). Incubation of L2 cells with only the supernatant from cultures of RB50 did not appear to cause cytotoxic effects.

To measure the cytotoxicity of various strains of *B. bronchiseptica* towards macrophage cell lines J774 and RAW, the release of lactate dehydrogenase was measured after a 4 hour incubation with bacteria. WD3 was significantly less cytotoxic to these cells than RB50 (Table 1). Therefore, cytotoxicity of *B. bronchiseptica* towards phagocytic and non-phagocytic mammalian cells *in vitro* depends, at least partially, on a functional BscN.

Wild type B. bronchiseptica, *but not the* bscN *deletion strain, causes tyrosine dephosphorylation of mammalian cell proteins when bound to L2 cells.*

One of the Yops secreted by the type III secretion system in *Yersinia* is YopH which has sequence similarity to mammalian protein-tyrosine phosphatases (PTP) and is injected into host cells upon contact, causing tyrosine dephosphorylation of specific host cell proteins (Andersson et al., 1996, Black and Bliska, 1997; Persson et al., 1997). To determine if *B. bronchiseptica* causes any change in tyrosine-phosphorylation of host cell proteins upon attachment, bacteria were bound to L2 cells for one hour, then total proteins were separated by SDS-PAGE and probed with antibody specific for phosphotyrosine. Figure 7, lane 1 shows that only one protein from *B. bronchiseptica* itself was recognized by the antibody while lane 2 shows a number of tyrosine phosphorylated polypeptides from uninfected L2 cells. Within one hour of binding to L2 cells, wild type Bvg^+ phase RB50 caused the tyrosine dephosphorylation of two or more high molecular weight polypeptides from the L2 cells (lane 3, arrows). This dephosphorylation could be inhibited in the presence of 1 mM vanadate (lane 4), which inhibits PTPs. This dephosphorylation process did not occur following the binding of Bvg^+ phase WD3 to L2 cells (lane 5) or FF1, the Bvg^- phase-locked strain expressing FHA adhesin (lane 6). Effector proteins secreted by *B. bronchiseptica* into host cells via the Bsc type III secretion system cause tyrosine dephosphoylation of specific host proteins.

B. bronchiseptic*a type III secretion system dependent apoptosis.*

Bvg+ phase *B. bronchiseptica* adhere to a variety of mammalian cells in culture, including rat lung epithelial (L2) cells and J774A.1 and RAW macrophage-like cells. Prolonged binding of RB50 to L2 cells (>2 hours) caused the L2 cells to show marked signs of cytotoxicity. Lactate dehydrogenase release measurements also support the cytotoxicity of RB50 to J774A.1 and RAW cells. None of these cells displayed signs of cytotoxicity when bound by FF1, a Bvg- phase-locked strain that binds the cells by virtue of an ectopically expressed FHA. In all three cell lines, cytotoxicity was shown to be dependent

on type III secretion, as WD3 induced less than 4% of the levels of cytotoxicity observed following incubation with RB50.

The cytotoxicity resulting from the activity of the type III secretion system is correlated with apoptosis. Fluorescent TUNEL reagent was used to detect DNA fragmentation, a characteristic of apoptotic cells (Fig. 15). At 30 min. post infection 30% of L2 cells and 60% of J774A.1 cells incubated with RB50 contained strongly labeled nuclei. In contrast, although L2 and J774A.1 cells incubated with WD3 had similar numbers of adherent bacteria, a negligible fraction of nuclei were stained in the TUNEL reaction. DNA fragmentation analysis showed similar results. Thus, wild type *B. bronchiseptica* can induce apoptosis in both phagocytic and non-phagocytic mammalian cells. A type III secretion effector or combination of effectors, is useful for inducing apoptosis in mucosal epithelial and immune cells.

B. bronchiseptic*a type III secretion system dependent NF-κB sequestration.*

NF-κB is a eukaryotic transcription factor that plays a central role in mediating gene expression induced by pathogens and other noxious stimuli. *NF-κB* is activated when it is released from *I-κB*, an inhibitory subunit which masks *NF-κB*'s nuclear localization signals. Upon translocation from the cytoplasm to the nucleus, *NF-κB* functions as a transcriptional activator for a variety of genes including those encoding inflammatory cytokines such as IL-6, IL-8, TNFα and GM-CSF. *NF-κB* has also been shown to play a role in preventing apoptosis, most likely by inducing the expression of "anti-apoptotic genes."

Our experiments using an anti- *NF-κB* antibody and immunofluorescence microscopy showed that type III secreted effector factors alter *NF-κB* activity. (Fig. 17.) In uninfected L2 cells, *NF-κB* staining was diffuse and evenly spread throughout the cytoplasm. Stimulation by TNFα resulted in intense nuclear staining, indicative of *NF-κB* translocation into the nucleus. In contrast, in cells infected with RB50, anti- *NF-κB* staining was concentrated in discrete cytoplasmic foci, suggesting *NF-κB* was localized in the cytoplasm in large complexes of unknown composition.

The pattern of *NF-κB* staining in WD3 infected cells was the same as in uninfected cells, i.e. diffusely localized throughout the cytoplasm, indicating that the aberrant localization of *NF-κB* seen in RB50 infected cells requires Bsc type III secretion. Furthermore, RB50, but not WD3, inhibits TNFα-mediated translocation of *NF-κB* to the nucleus.

Type III secretion is necessary for long term tracheal colonization in rats.

Wistar rats inoculated with low doses of RB50 (ID_{50}<20) become chronically infected in the nasal cavity and trachea (Akerley et al., 1995; Martinez de Tejada et al., 1996). To determine if WD3 is capable of colonizing the rat respiratory tract, 1000 colony forming units (cfu) of either RB50 or WD3 were inoculated intranasally in a 5 µl droplet into groups of Wistar rats (female, 4 weeks old). After 14 days, similar numbers of cfu were recovered from the nasal septum and trachea of all rats infected with either strain. After 35 days of infection, similar numbers were recovered from the nasal septum of rats infected with either strain. WD3, however, was not found in the trachea of infected animals while all rats infected with RB50 had >10^5/centimeter bacteria recovered from the trachea (Fig. 8; see also Fig. 11a and b for other mutants and other species.). Therefore, WD3 appears to be specifically defective in persistence in the trachea, but not in establishing infection or in long-term colonization of the nasal septum. The rather remarkable specificity of the infection phenotype may relate to the fact that the trachea is usually sterile, whereas the nasal cavity is teaming with bacteria. bscN-dependent secretion may therefore be required to evade inducible immune responses that normally protect the lower respiratory tract.

Examination of lung tissue from wild type mice 24 hours post-inoculation with 10^5 cfu of wild type *B. bronchiseptica* delivered in a 50 µl volume showed a modest degree of inflammation. Many of the inflammatory cells that were present appeared to be undergoing apoptosis as indicated by strong nuclear staining with the fluorescent TUNEL reagent. In contrast, lungs from mice infected with WD3 showed a significantly greater degree of inflammation and very few of the cells stained positively with the TUNEL reagent. Fig. 16.

Comparison of bscN *expression in* Bordetella spp.

A primer pair specific for the sequence of *bscN* in *B. bronchiseptica* strain RB50 (W3+W4) were used for PCR amplification of genomic DNA and cDNA from the following strains of *Bordetella*: *B. pertussis* strains Tohama 1, 18323, GMT1 and 17471, *B. parapertussis* strains A168 (human isolate) and H 1 (ovine isolate). All of the strains tested contained the genomic sequence for *bscN* as shown by amplification of an expected 420 bp band from genomic DNA (Fig. 9). Amplification from cDNAs showed that *B. pertussis* strain 18323 and the ovine isolate of *B. parapertussis* produced a detectable transcript from this gene. A very weak signal was detected from *B. pertussis* strain 17471, while Tohama 1 and GMT 1 did not produce any detectable RT-PCR product. Other primers specific for

sequences in the *bscJ*, *bscK* and *bscL* loci also gave the same results (i.e. fragments of the expected size from genomic DNA but no products from cDNA). Controls using primers specific for *fhaB* showed that the cDNA preparations were Bvg^+ phase specific. These results indicate that type III secretion genes are present in all the tested *Bordetella* strains. However, under the in vitro conditions used to grow the bacteria for RNA isolation, *B. bronchiseptica* strain RB50, *B. pertussis* strain 18323 and ovine *B. parapertussis* strain H1 showed significant transcription of *bscN* detectable by RT-PCR, while the other *B. pertussis* strains and the human *B. parapertussis* isolate did not.

Generation of genetically engineered, heterologous antigen producing Bordetella.

DNA encoding the heterologous *Pasteurella multocida* antigen, is subcloned between two flanking sequences of *Bordetella* DNA. The flanking sequences are preferably about 500 bps. If the a fusion protein is desired, the flanking sequences are part of the open reading frame in which the antigen will be fused to such that all the sequences are in the same reading frame. The construct is then cloned into a suitable suicide vector, having a selectable marker (antibiotic resistance, for example) and a counter-selectable marker (sucrose sensitivity, for example). pEGBR, for example, may be used. The vector is then transformed into suitable bacteria, E. coli strain SM10, for example. The allelic exchange vector is then transferred into Bordetella by conjugation. *Bordetella* that has received the plasmid and integrated it into the genome at the specific site (determined by the flanking sequences) can be selected by antibiotic resistance encoded by the suicide plasmid. This strain of *Bordetella* is then counter-selected with sucrose to select clones that have undergone a second recombination event to remove the integrated plasmid. Colonies that have the desired antigen DNA sequence integrated into their genome can be identified by PCR with specific primers, southern hybridization or immunoblots with antibodies specific for the antigen, or the like.

For example, an allelic exchange vector with the open reading frame for the desired gene may be inserted in the SnaB1 site between the end of the *B. bronchiseptica* flaA coding region and immediately upstream of the transcription stop site. This construct does not disrupt expression of flaA and does not affect expression of downstream genes. Other constructs using other genes may also be used.

Live attenuated Bordetella *are effective protecting hosts from infection by wild type* B. bronchiseptica.

Eight rats per group were inoculated with 10^4 cfu of either wild type *B. bronchiseptica* (RB50) or the *bscN* mutant (WD3). 35 days later, three rats from each group were sacrificed and colonization levels in the nasal cavities and tracheas were determined. As expected, RB50 was recovered at about 10^5 cfu from each nasal septum and about 10^{4-6} cfu per cm trachea, while WD3 was also recovered at about 10^5 cfu per nasal septum but no WD3 bacteria were recovered from the tracheas. The remaining five rats were challenged with 10^6 cfu of RB50G delivered in a 50µl volume. (RB50G is a derivative of RB50 containing a Gm resistance gene immediately 3' to the *fla*A gene. This strain is indistinguishable from RB50 in its ability to establish persistent infections in rats and mice). A 50µl volume was used for the challenge so that bacteria are delivered to the entire respiratory tract (i.e. the nasal cavity, the trachea and the lungs). Seven days later, the rats were sacrificed and the number of Gm^s and Gm^r bacteria recovered from the nasal septa, larynx, trachea and lungs determined. For rats initially infected with RB50, 2 of the 5 rats contained low numbers (about 10^2) of Gm^r bacteria in their nasal cavities and tracheas while no Gm^r bacteria were recovered from any site in the remaining 3 rats. For animals initially infected with WD3, no Gm^r bacteria were recovered from any site in the respiratory tract from any animal. The number of Gm^s bacteria (RB50 and WD3) recovered from each site was similar to those rats sacrificed before challenge. The attenuated strain, WD3, was therefore superior to wild type *B. bronchiseptica* in its ability to induce protective immunity against wild type *B. bronchiseptica*.

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Claims

1. A genetically engineered type III secretion system *Bordetella* mutant.

2. The type III secretion system mutant of claim 1 comprising a mutation in a gene encoding a protein or polypeptide selected from the group consisting of core component, effector, accessory factor and combinations thereof.

3. The type III secretion system mutant of claim 2, the mutation being present in *Bordetella* selected from the group consisting of *B. pertussis*, *B. bronchiseptica* and *B. parapertussis*.

4. The type III secretion system mutant of claim 3, the mutation being present in *B. bronchiseptica*.

5. The type III secretion system mutant of claim 2, the mutation being present in gene encoding a protein or polypeptide selected from the group consisting of bscV, bcr3, bopN, bsp22, bcrH1, bopD, bopB, bcrH2, bcr4, bscl, bscJ, bscK, bscL, bscN, bscO and combinations thereof.

6. The type III secretion system mutant of claim 5, the mutation being present in the gene encoding bscN.

7. The type III secretion system mutant of claim 5, the mutation being present in the gene encoding bsp22.

8. The type III secretion system mutant of claim 1, the mutation comprising the deletion of a type III secretion system protein locus.

9. The type III secretion system mutant of claim 1, the mutation comprising an in frame codon deletion of a type III secretion system protein locus.

10. The type III secretion system mutant of claim 1, the mutation comprising a codon substitution of a type III secretion system protein locus.

11. A live, attenuated vaccine component against *Bordetella* comprising a type III secretion system mutant of claim 1.

12. The live, attenuated vaccine component against *Bordetella* of claim 11 comprising a mutation in a gene encoding a protein or polypeptide selected from the group consisting of core component, effector, accessory factor and combinations thereof.

13. The live, attenuated vaccine component against *Bordetella* of claim 11, the mutation being present in a *Bordetella* selected from the group consisting of *B. pertussis*, *B. bronchiseptica* and *B. parapertussis*.

14. The live, attenuated vaccine component against *Bordetella* of claim 13, the mutation being present in *B. bronchiseptica.*

15. The live, attenuated vaccine component against *Bordetella* of claim 12, the mutation being present in gene encoding a protein or polypeptide selected from the group consisting of bscV, bcr3, bopN, bsp22, bcrH1, bopD, bopB, bcrH2, bcr4, bscl, bscJ, bscK, bscL, bscN, bscO and combinations thereof.

16. The live, attenuated vaccine component against *Bordetella* of claim 15, the mutation being present in the gene encoding bscN.

17. The live, attenuated vaccine component against *Bordetella* of claim 15, the mutation being present in the gene encoding bsp22.

18. The live, attenuated vaccine component against *Bordetella* of claim 11, the mutation comprising the deletion of a type III secretion system protein locus.

19. The live, attenuated vaccine component against *Bordetella* of claim 11, the mutation comprising an in frame codon deletion of a type III secretion system protein locus.

20. The live, attenuated vaccine component against *Bordetella* of claim 11, the mutation comprising a codon substitution of a type III secretion system protein locus.

21. A purified nucleic acid comprising a sequence encoding a *Bordetella* type III secretion system component.

22. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 1.

23. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 2.

24. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 3.

25. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 4.

26. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 5.

27. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 6.

28. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 7.

29. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 8.

30. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 9.

31. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 10.

32. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 11.

33. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 12.

34. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 13.

35. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 14.

36. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 15.

37. The purified nucleic acid of claim 21, the sequence comprising Seq. ID 16.

38. The purified nucleic acid of claim 21, the sequence being selected from the group consisting of Seq. ID 1, Seq. ID 2, Seq. ID 3, Seq. ID 4, Seq. ID 5, Seq. ID 6, Seq. ID 7, Seq. ID 8, Seq. ID 9, Seq. ID 10, Seq. ID 11, Seq. ID 12, Seq. ID 13, Seq. ID 14, Seq. ID 15 and Seq. ID 16.

39. An isolated and purified polypeptide encoded by a nucleic acid sequence selected from the group consisting of Seq. ID 2, Seq. ID 3, Seq. ID 4, Seq. ID 5, Seq. ID 6, Seq. ID 7, Seq. ID 8, Seq. ID 9, Seq. ID 10, Seq. ID 11, Seq. ID 12, Seq. ID 13, Seq. ID 14, Seq. ID 15 and Seq. ID 16.

40. A live mucosal antigen-delivery vector comprising a genetically engineered *Bordetella* expressing a heterologous antigen.

41. The live mucosal antigen-delivery vector of claim 40, wherein the heterologous antigen comprises a protective epitope.

42. The live mucosal antigen-delivery vector of claim 40 wherein the heterologous antigen is derived from an agent selected from the group consisting of *Leptospira canicola, L. grippotyphosa, L. hardjo, L. icterohaemorrhagiae, L. pomona, L. interrogans, L. bratislava*, canine distemper virus, canine adenovirus type 2, canine parainfluenza virus, canine parvovirus, rabies, herpes viruses, HIV, SIV, *Erysipelothrix rhusiopathiae, Pasteurella, P. multocida, Ascaris, Oesophagostomum*, pseudorabies virus, porcine parvovirus, pathogenic *E. coli*, including *E. coli* having K88, K99, 987P, and/or F41 adherence factors, *Clostridium spp.*, including *Cl. perfringens*, and *Cl. perfringens* type C beta toxoid, *Salmonella, Vibrio, Mycoplasma, Actinobacillus pleuropneumoniae, Haemophilus*, rotavirus, transmissible gastroenteritis virus, *Streptococcus sobrinus, S. mutans*, and *Bordetella* virulence factors.

43. The live mucosal antigen-delivery vector of claim 42 wherein the heterologous antigen is *Pasteurella multocida*.

44. The live mucosal antigen-delivery vector of claim 40 wherein the genetically engineered *Bordetella* further comprises a type III secretion system mutant.

45. The live mucosal antigen-delivery vector of claim 44 wherien the genetically engineered *Bordetella* is *Bordetella bronchiseptica*.

46. A vaccine comprising the live mucosal antigen-delivery vector of claim 40.

47. A method for vaccinating an animal susceptible to infection by wild type *Bordetella bronchiseptica* comprising administering an effective amount of a genetically engineered *Bordetella bronchiseptica* comprising a type III secretion system mutant.

48. A method for vaccinating an animal susceptible to infection by an agent comprising administering an effective amount of a *Bordetella* genetically engineered to express an antigen of the agent.

49. A method for cloning a DNA molecule having a sequence encoding a protein or polypeptide secreted by a *Bordetella* type III secretion system comprising isolating and characterizing a protein or polypeptide secreted into the medium by wild type *Bordetella* but not by a *Bordetella* type III secretion mutant.

50. The method of claim 49 wherein the method is selected from the group consisting of:

a) 1) culturing a *Bordetella* type III secretion mutant which does not secrete type III secretion factors;

2) isolating the supernatant of the mutant culture;

3) precipitating and separating the proteins in the supernatant;

4) performing steps 1-3 with a culture of wild type *Bordetella*;

5) isolating polypeptides present in the wild type but absent from the mutant culture supernatant;

6) determining the amino-terminal sequences of the isolated polypeptide;

7) isolating a DNA sequence comprising codons encoding the determined amino-terminal sequence; and

b) 1) immunizing a first species with a supernatant from wild type *Bordetella* culture;

2) immunizing a second species with a supernatant from a *Bordetella* type III secretion mutant which does not secrete type III secretion proteins or polypeptides;

3) expression screening filters comprising a *Bordetella* expression library by first blocking the filters with a solution comprising the serum from species B, then hybridizing the filters with a solution comprising the serum of species A;

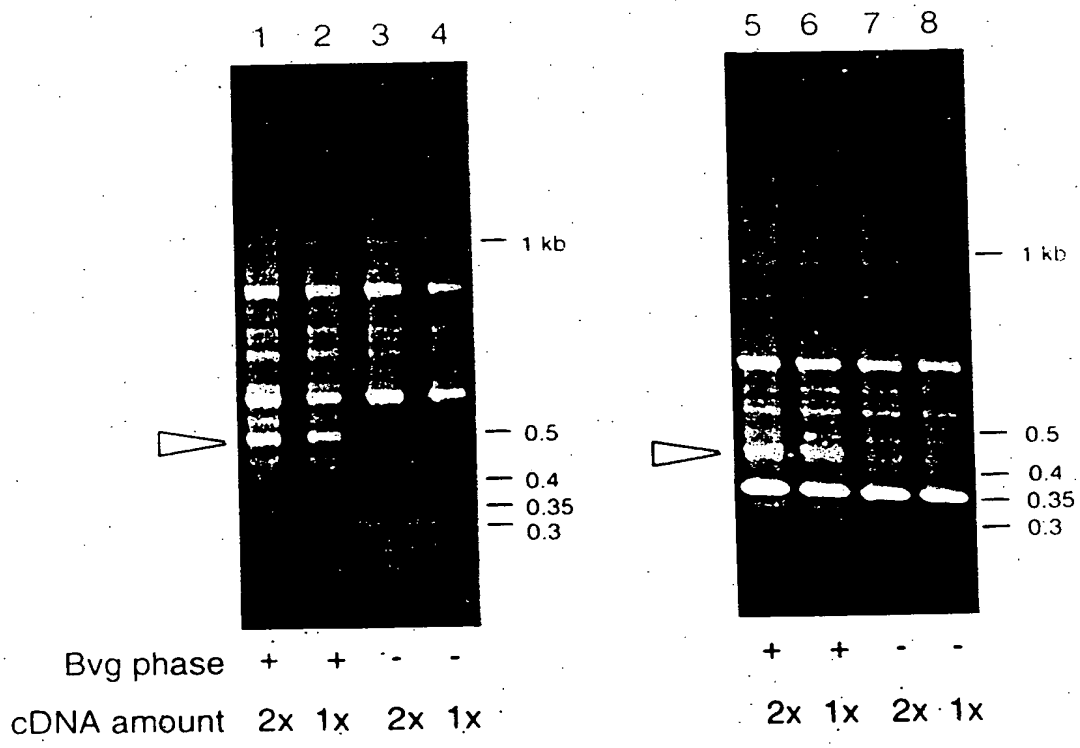
4) incubating the filters with a solution comprising labeled antibody specific for species A antibodies;

5) isolating a characterizing the clones obtained.

51. A DNA molecule encoding a protein or polypeptide secreted by a Bordetella type III secretion system obtained by the method of claim 49.

52. A DNA molecule encoding a protein or polypeptide secreted by a Bordetella type III secretion system obtained by the method of claim 50.

FIG. 1.



```
BscN    MRQYHYITEMMRVALQDLSTLRIKGRVVQVVGTIIKAVVPMVKIGEVCLLRNPGEDFEMH
        : .  : . .: ..  .:::::::::::::::::: :::::: :::: . . ..
YscN    MLSLDQIPHHIRHGIVGSRLIQIRGRVTQVTGTLLKAVVPGVRIGELCYLRNPDNSLSLQ

        GEVVGFVRDAALLTPIGDMYGISSATEVIPTGRTHMVPVGPGLLGRVLDGMGRPLDVAES
        .::::    ::: :.:::::::: ::: :::  :.: ::  :::.:::::.:.:    .
        AEVIGFAQHQALLIPLGEMYGISSNTEVSPTGTMHQVGVGEHLLGQVLDGLGQPFD---G

                                                        Walker Box A
        GPL-HAHKFYPVFADAPDPLTRRIIHAPLELGVRVLDGLLTCGEGQRLGIFAAAGGGKST
        : : .   .:::. ::: :.:.:.: .:: :::::::::::::::.:::::::::::
        GHLPEPAAWYPVYQDAPAPMSRKLITTPLSLGIRVIDGLLTCGEGQRMGIFAAAGGGKST

        LLGMLVKGAAVDVTVVALIGERGREVREFLEHELGPEGRRYSVIVCATSDKSSMERAKAA
        ::. :...: ::::::::::::: :::::: .:: :: ::. .: :::: :::::::.
        LLASLIRSAEVDVTVLALIGERGPEVREFIESDLGEEGLRKAFLVVATSDRPSMERAKAG

                    Walker Box B
        YVATAIAEYFRDQGQRVLFRMDSVTRFARAQREIGLAAGDPPTRRGYPPSVFATLPKLME
        .:::.::::::::.:::. ::::::::::::::::::::::::::::::::.::.:::
        FVATSIAEYFRDQGKRVLLLMDSVTRFARAQREIGLAAGEPPTRRGYPPSVFAALPRLME

        RAGMNQTGSITALYTVLVEGDDMNEPVADETRSILDGHIVLSRKLGAANHYPAVDVLASA
        :::... ::::::::::::::::::::::::::::::::::::.:::::.::::::.::: ::
        RAGQSSKGSITALYTVLVEGDDMTEPVADETRSILDGHIILSRKLAAANHYPAIDVLRSA

        SRVMNAVVSPRHKYLAGRMRELMAKYQDVELLVKIGEYKQGADASTDEAIQKIGQINAFL
        ::::: .:: .::  ::..:.:::.:::::.::::.: :  .:.::...: : ..:
        SRVMNQIVSKEHKTWAGHLRRLLAKYEEVELLLQIGEYQKGQDKEADQAIERMGAIRGWL

                                    444
        RQLTDEREAFEDTVLRMAEIIGPES
         : : :  :..: : . : .  .
        CQGTHELSHFNET-LNLLETL--TQ
                                   439
```

Fig 2

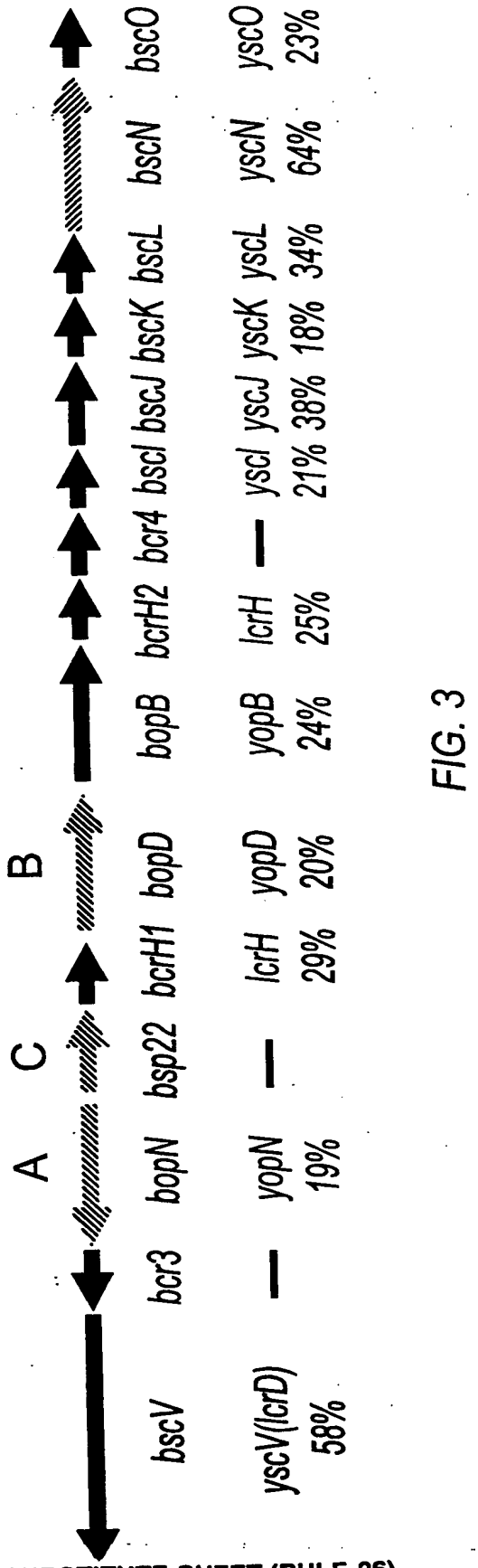


FIG. 3

FIG. 4A.

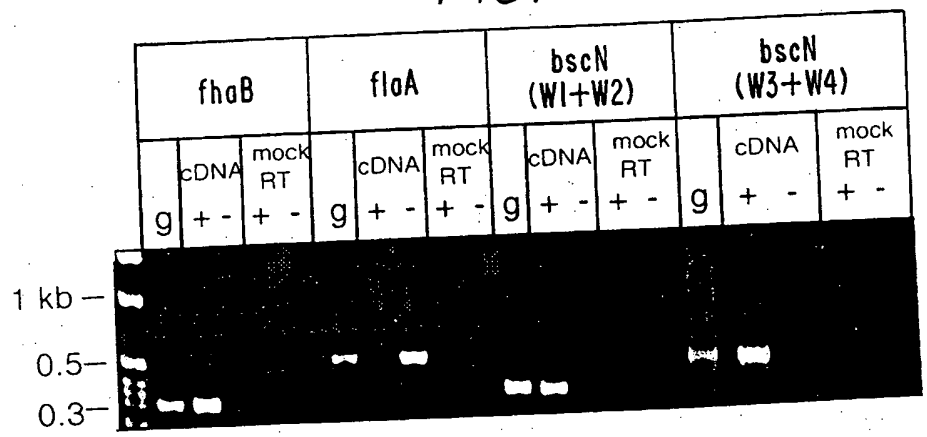


FIG. 4B.

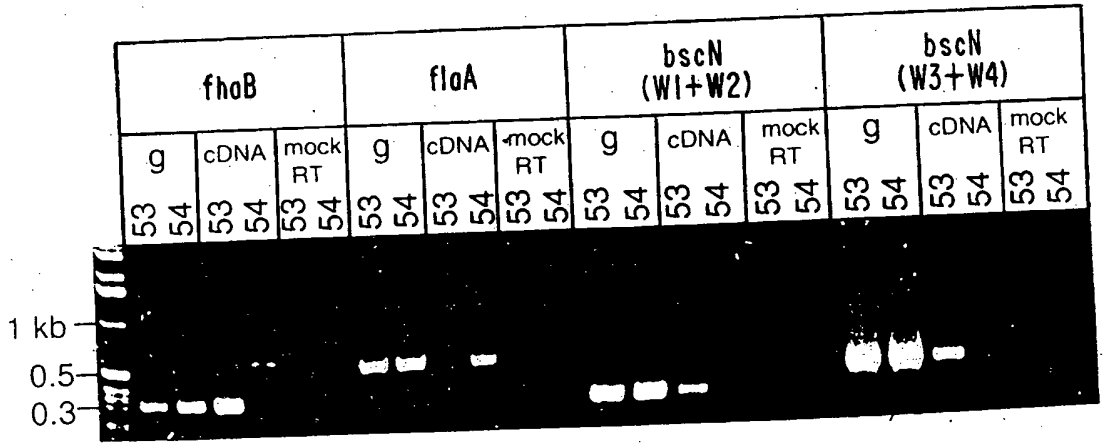


FIG. 4C.

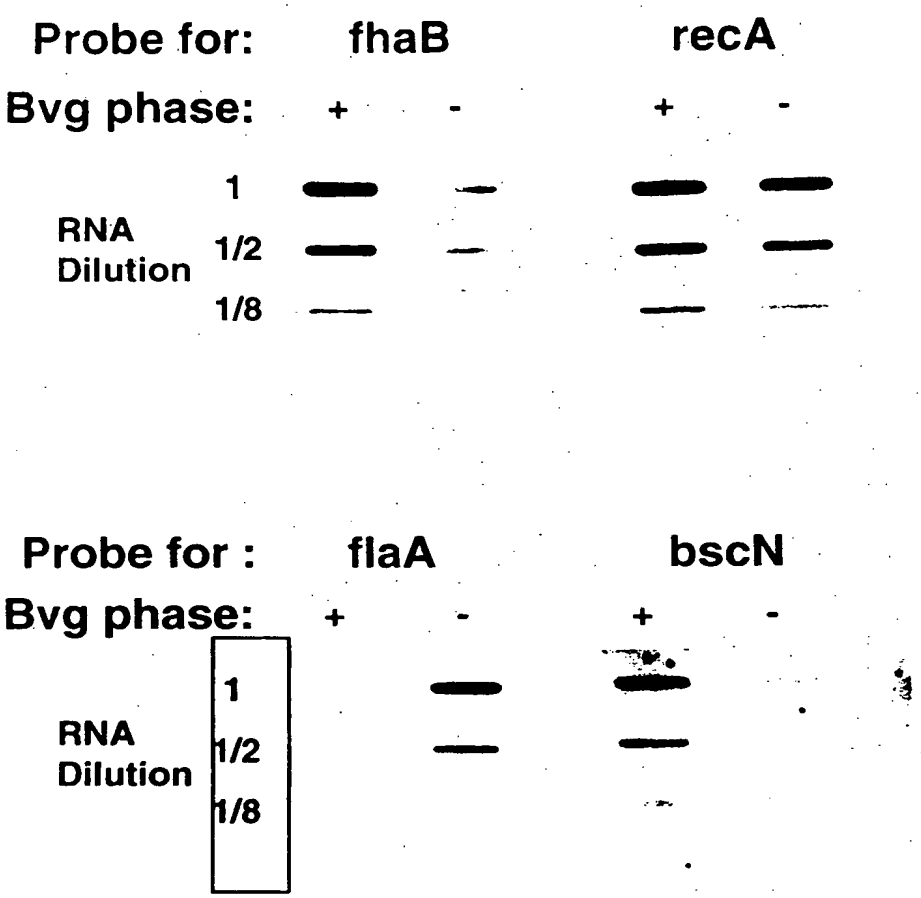


FIG. 5.

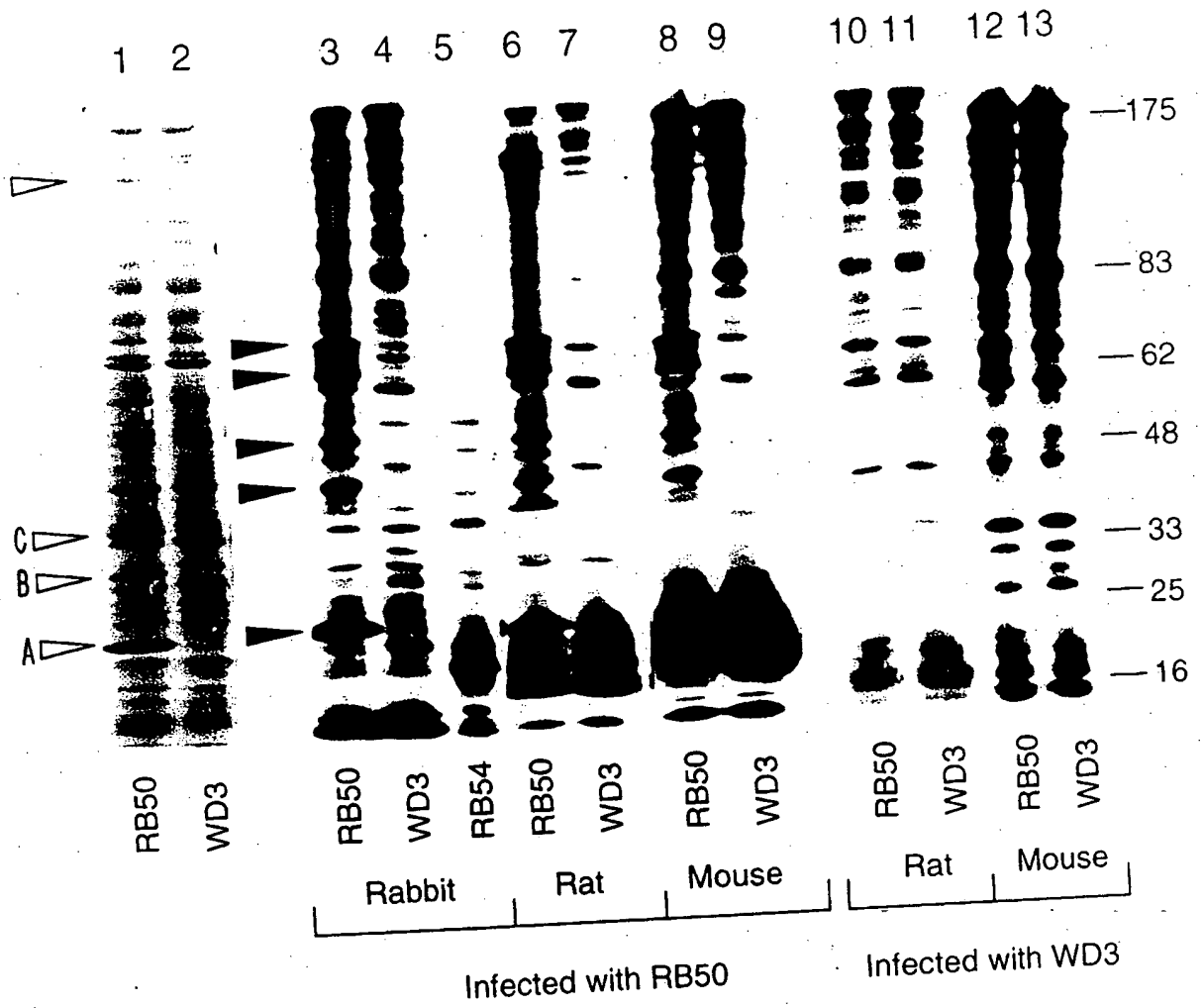


FIG. 5'

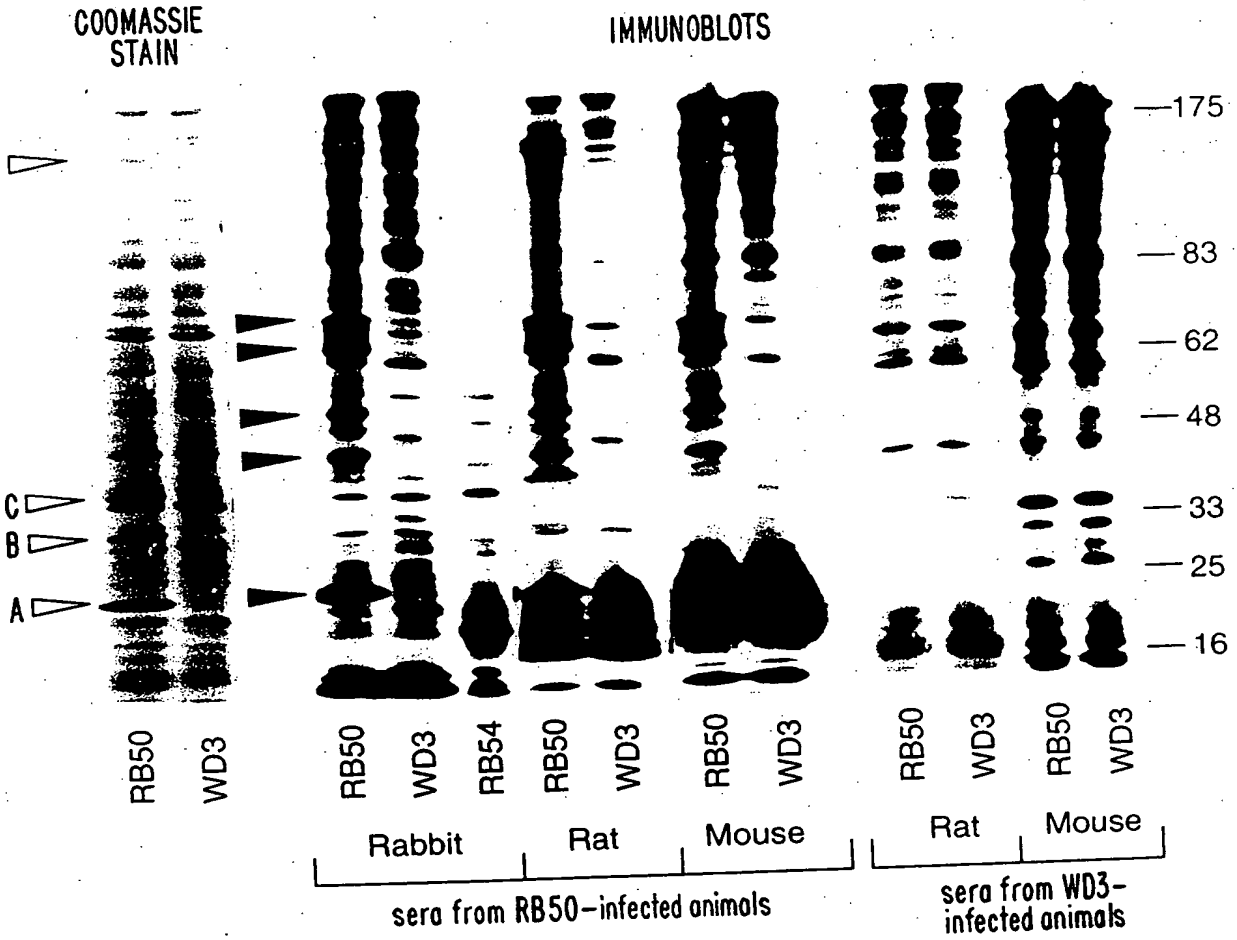

COOMASSIE STAIN
IMMUNOBLOTS
C
B
A
175
83
62
48
33
25
16
RB50
WD3
RB50
WD3
RB54
RB50
WD3
RB50
WD3
RB50
WD3
RB50
WD3
Rabbit
Rat
Mouse
Rat
Mouse
sera from RB50-infected animals
sera from WD3-infected animals

FIG. 6A.

FIG. 6B.

FIG. 6C.

FIG. 6D.

FIG. 6E.

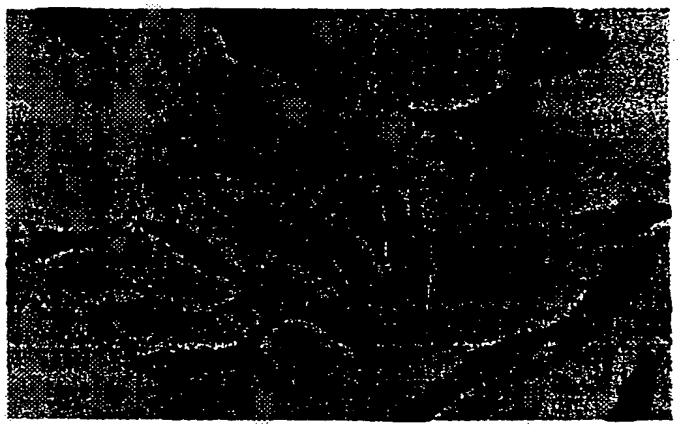
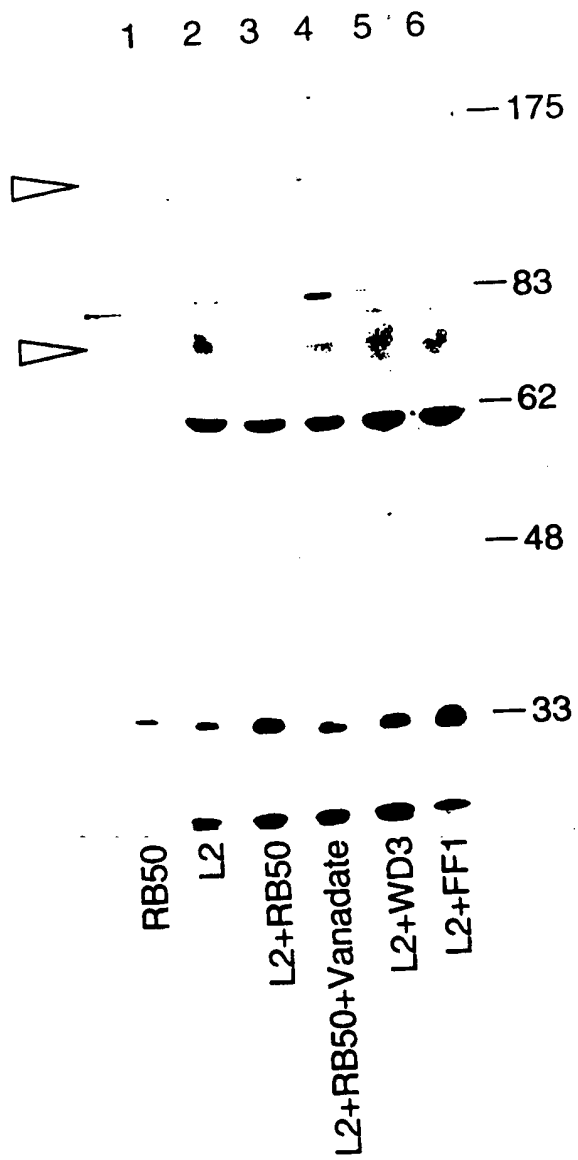


FIG. 6F.

FIG. 7.



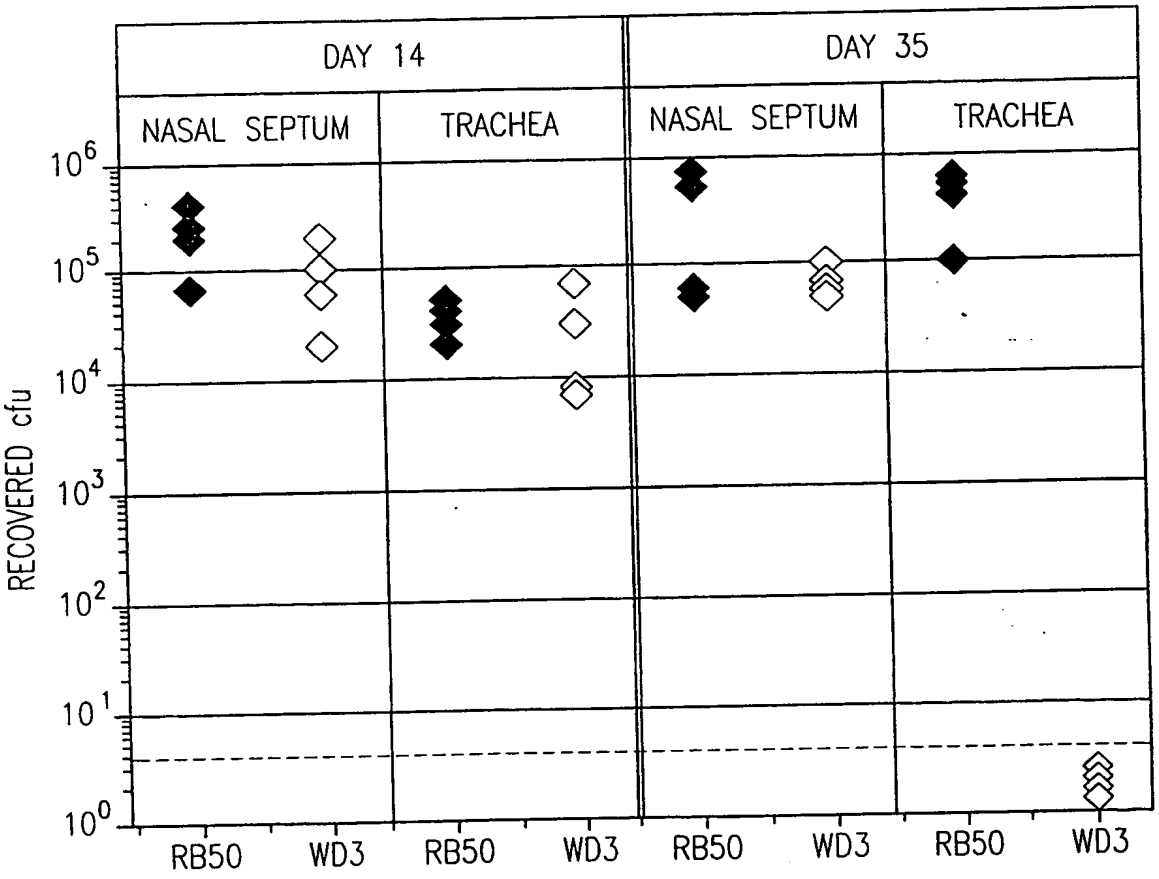


FIG. 8

FIG. 9.

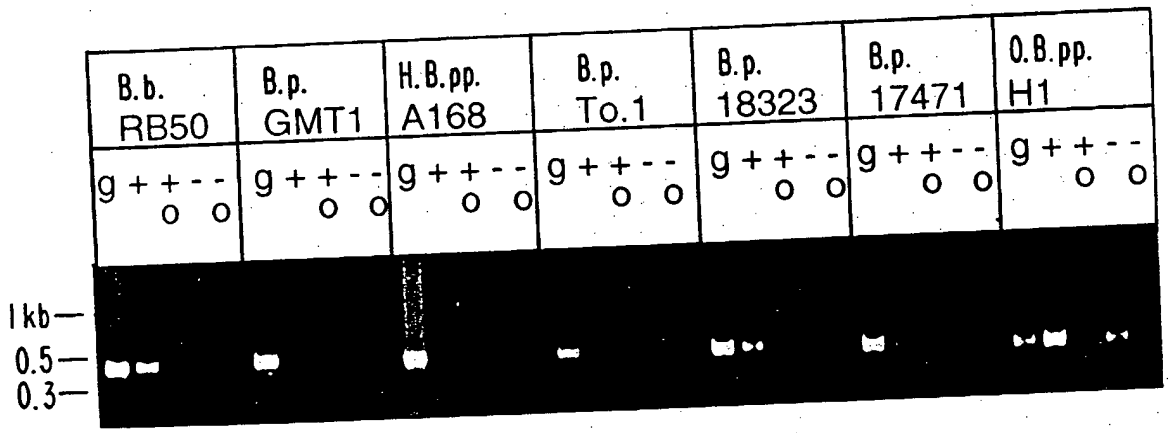


FIG. 10B.

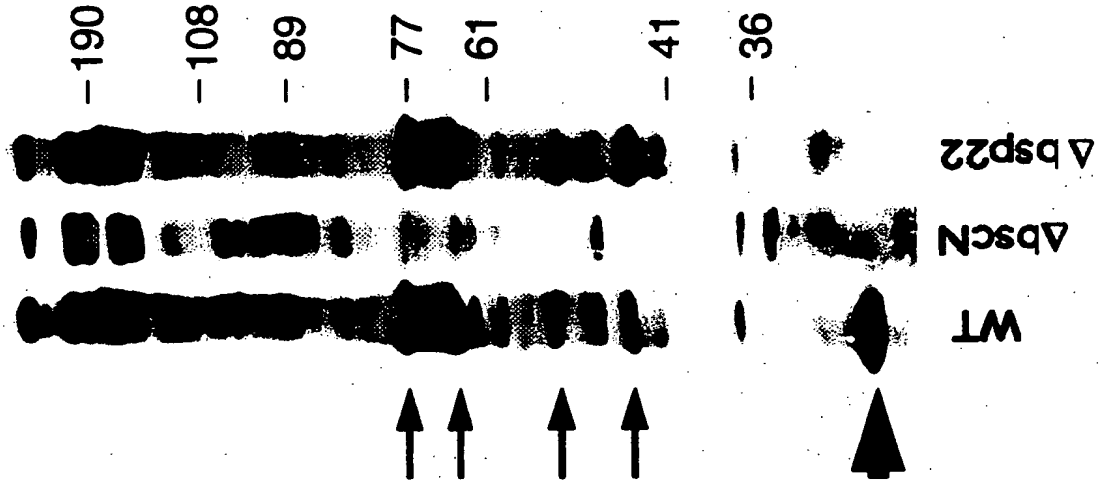
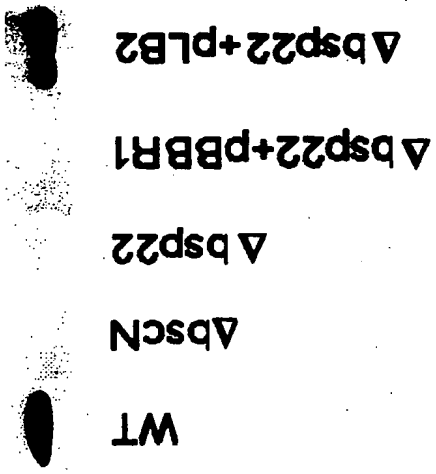


FIG. 10A.



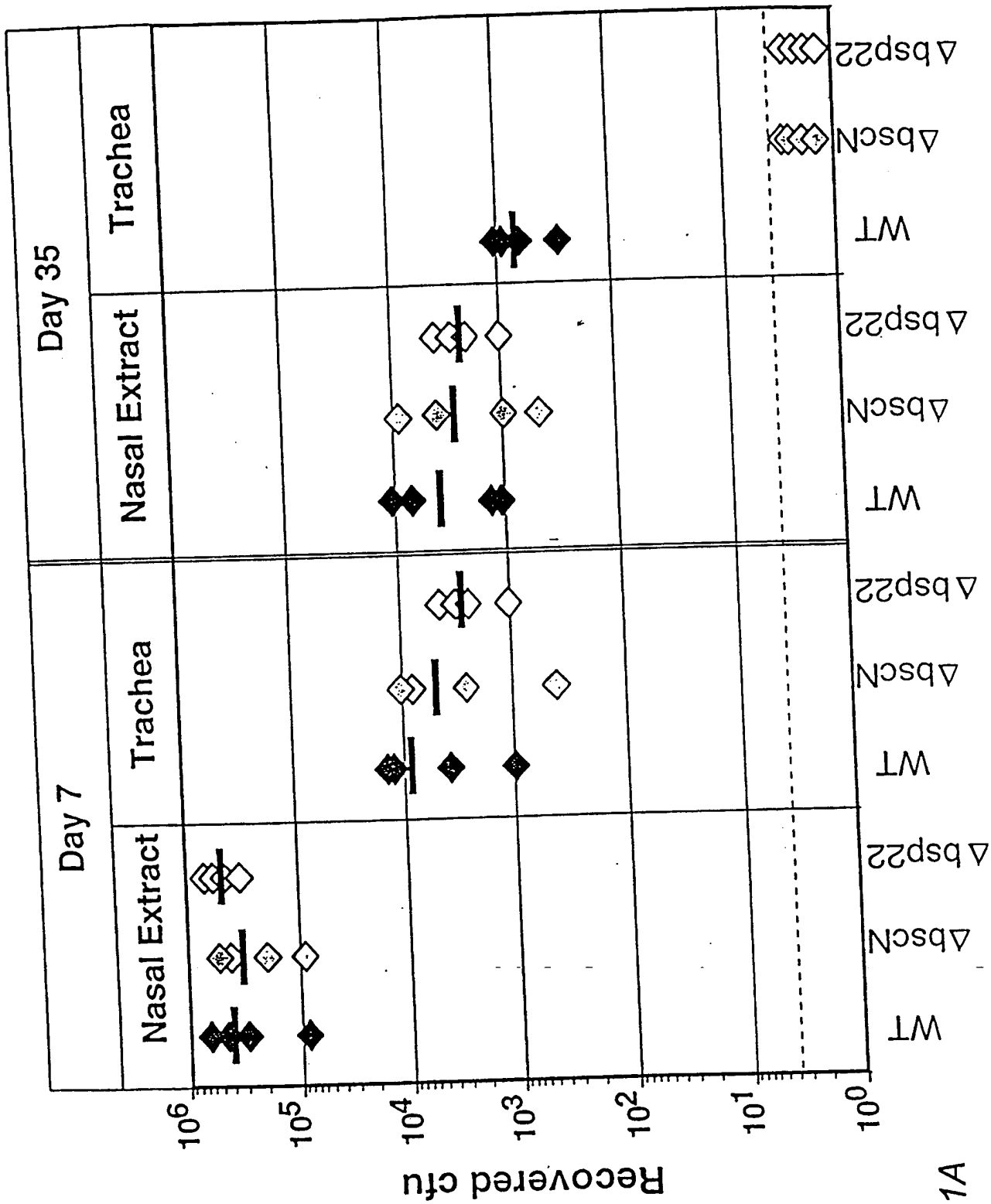


FIG. 11A

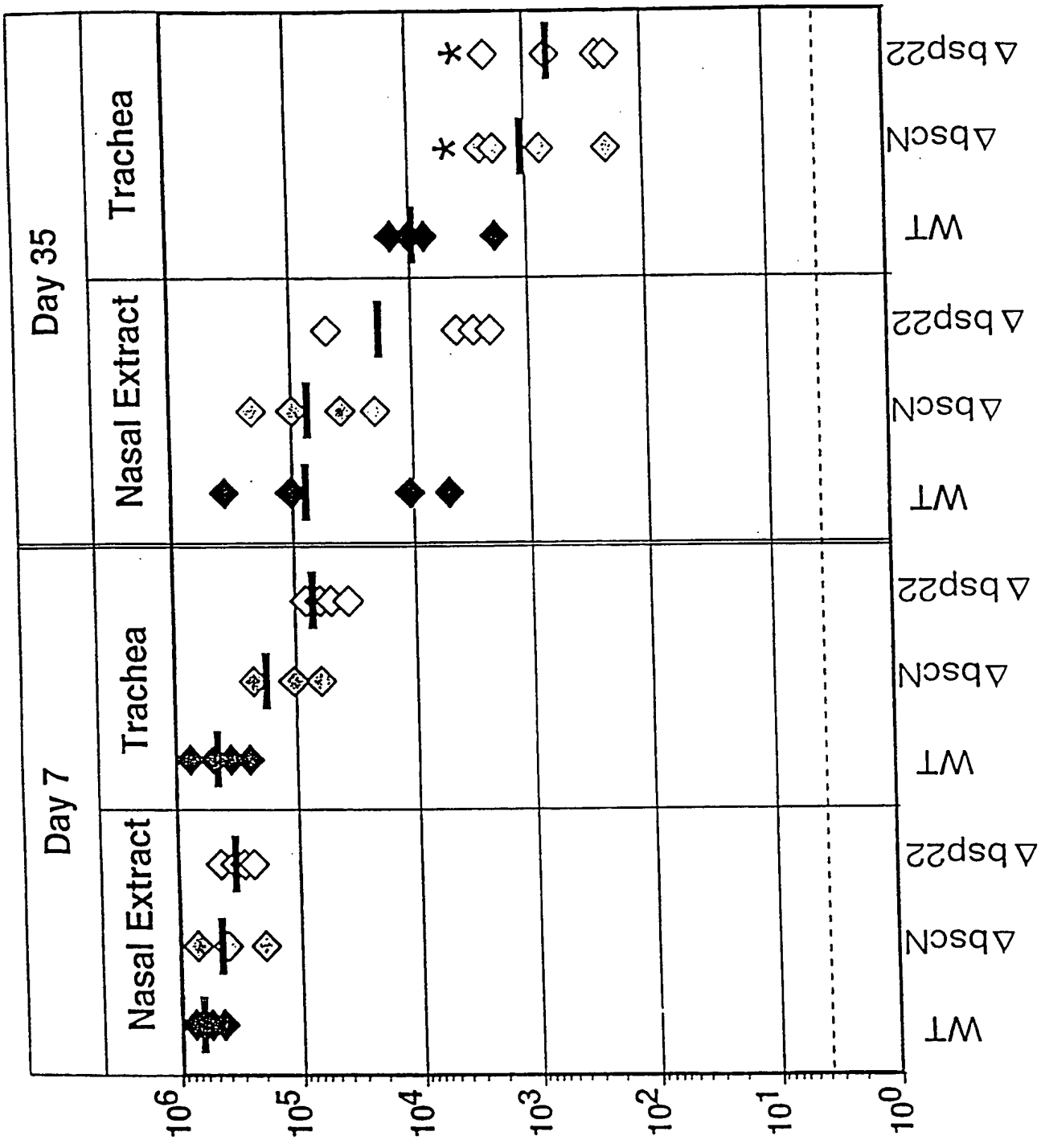


FIG. 11B

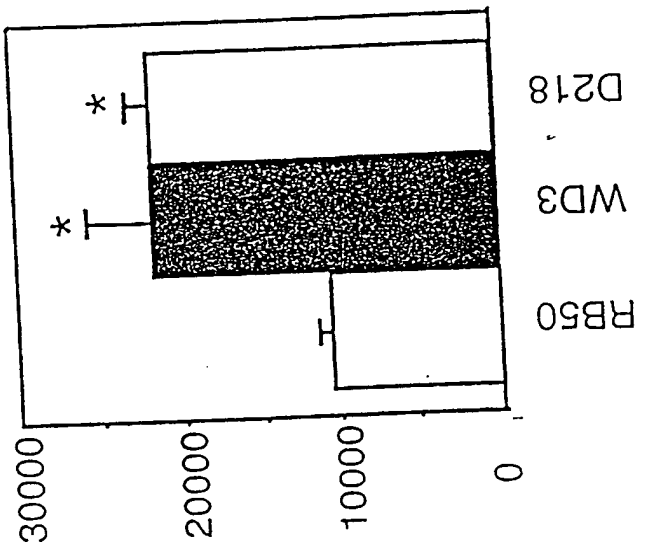


FIG. 12B

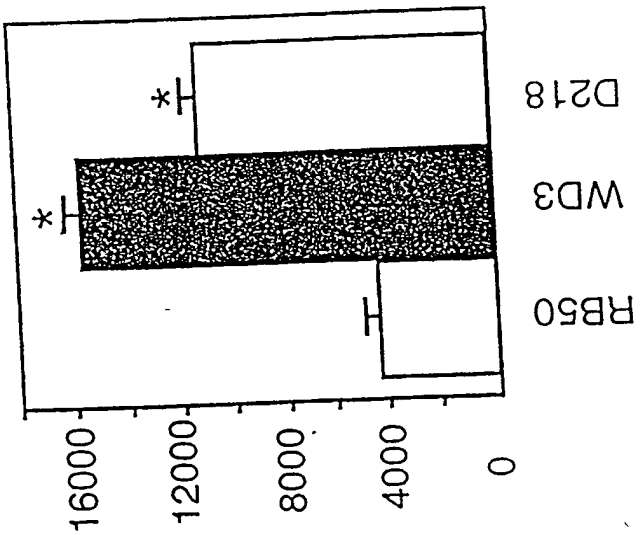


FIG. 12A

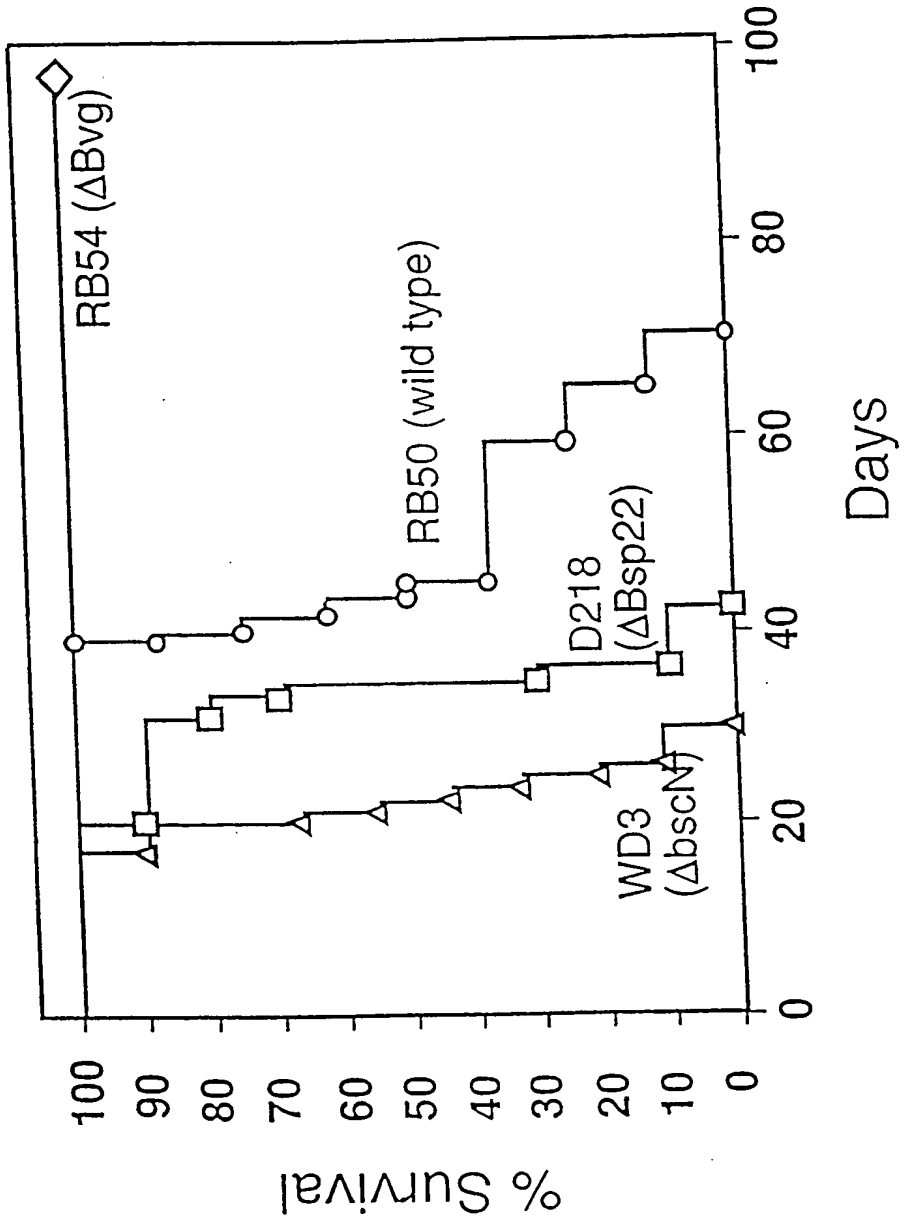


FIG. 13A

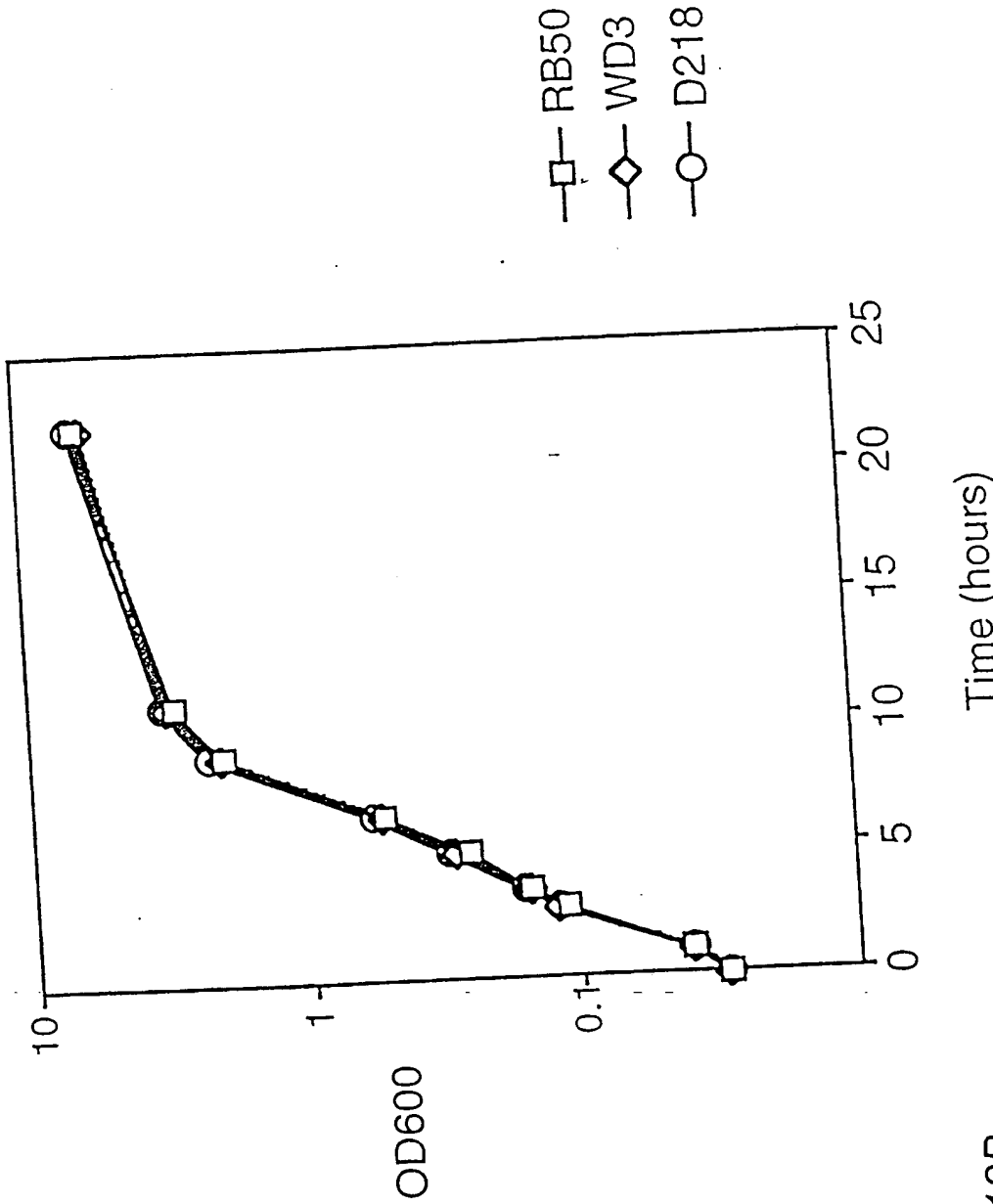


FIG. 13B

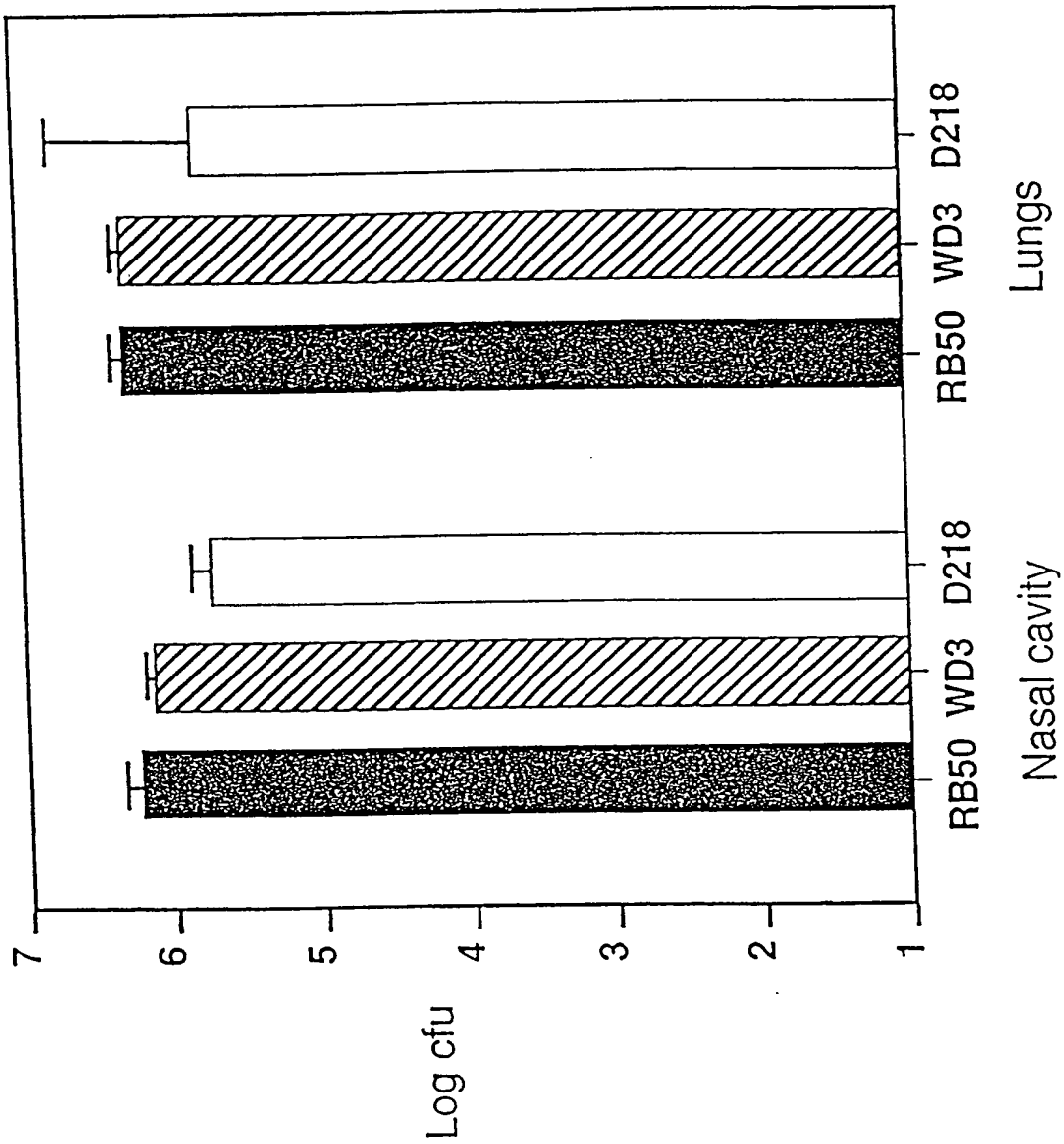


FIG. 13C

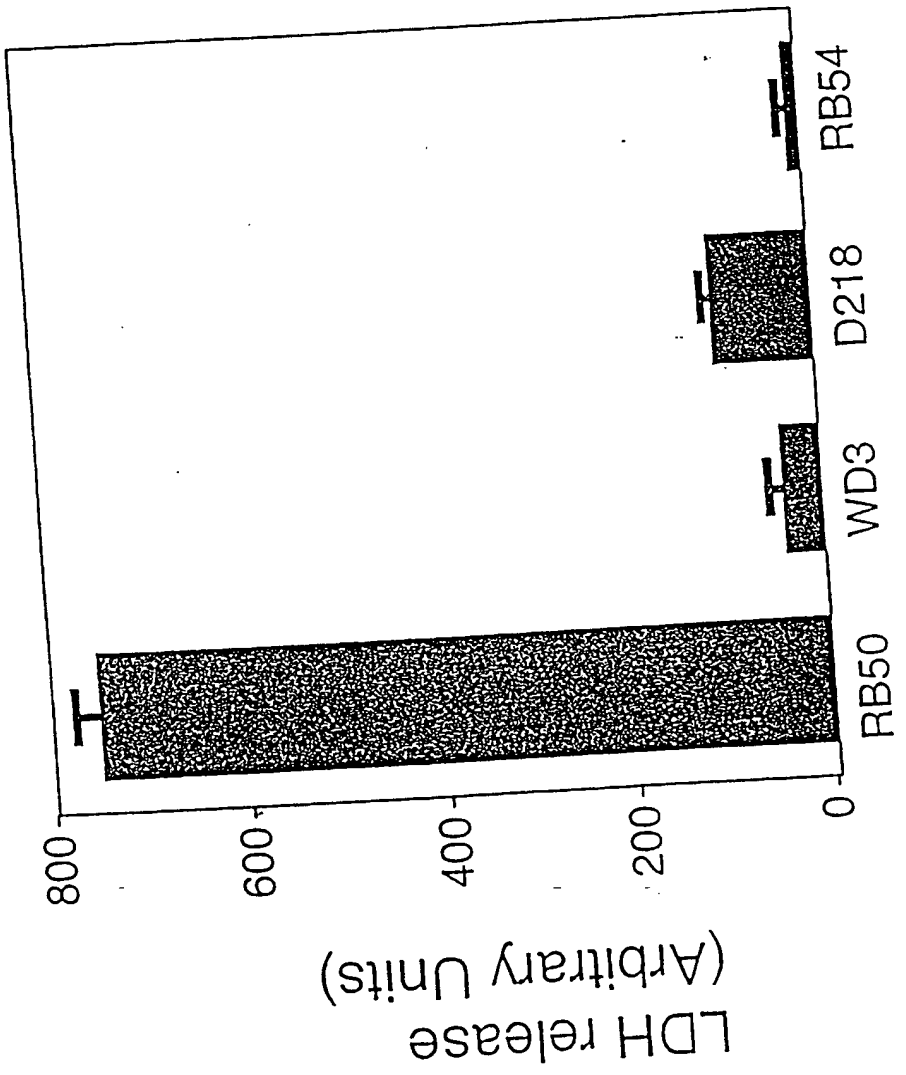


FIG. 14

FIG. 15C.

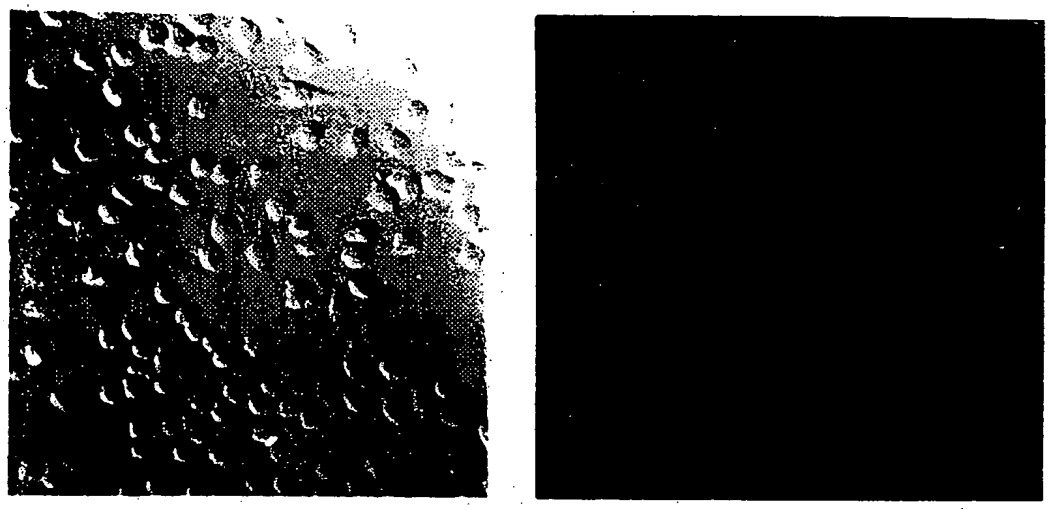


FIG. 15B.

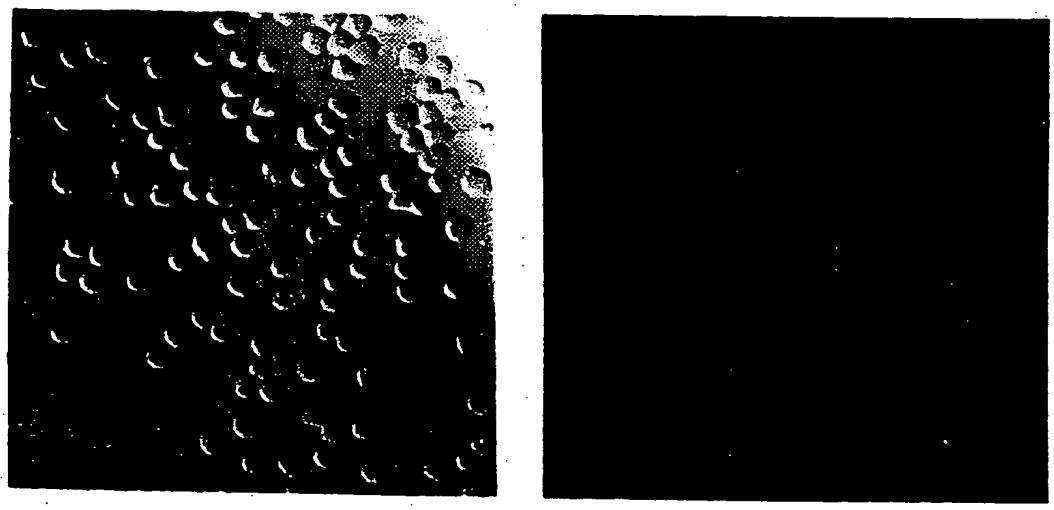


FIG. 15A.

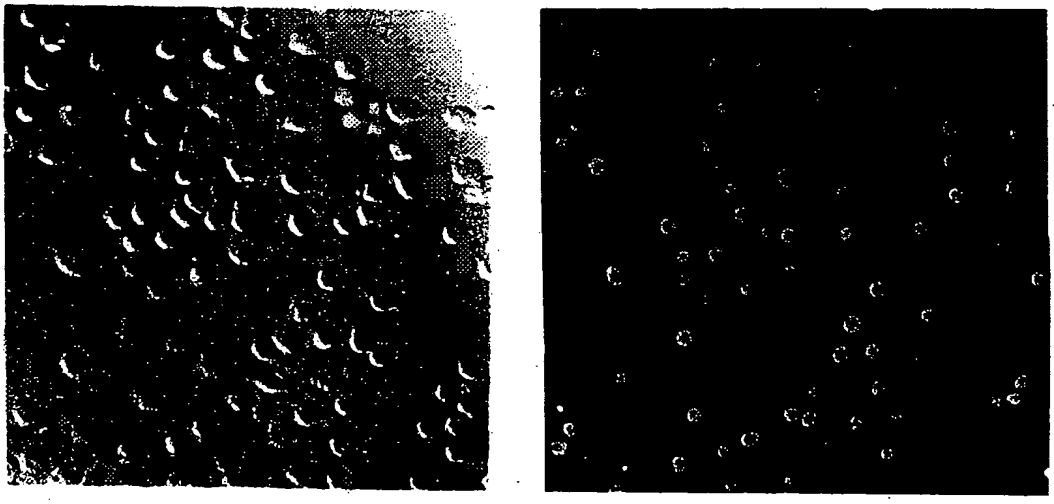


FIG. 16A.

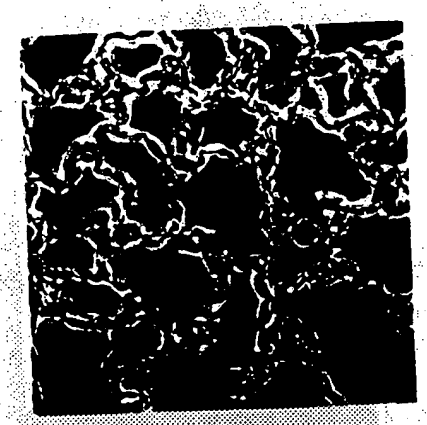


FIG. 16B.

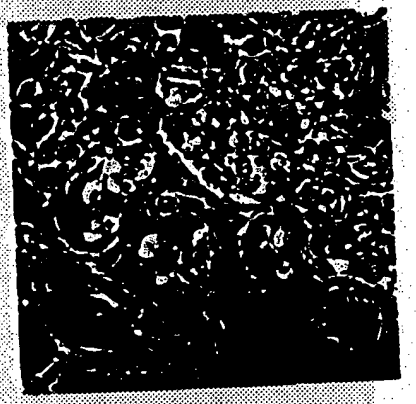
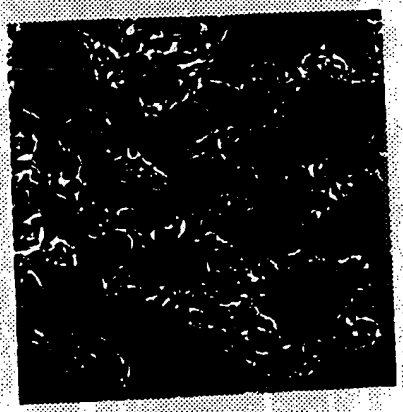


FIG. 16C.



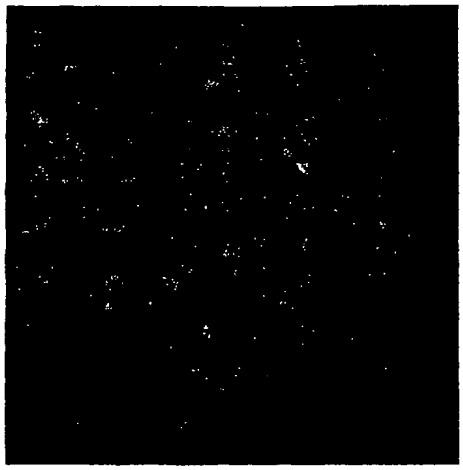


FIG. 17E.

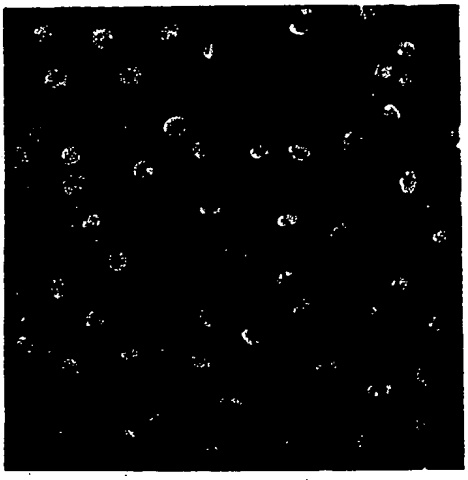


FIG. 17F.

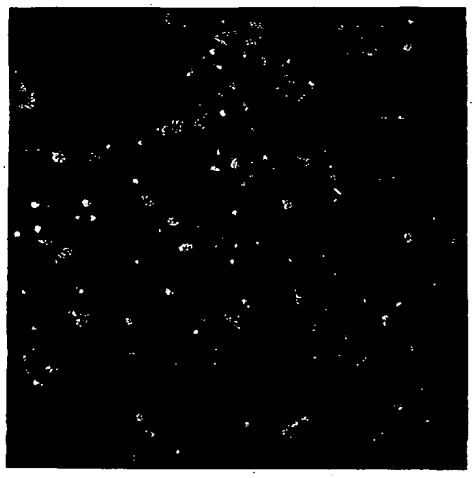


FIG. 17C.

FIG. 17D.

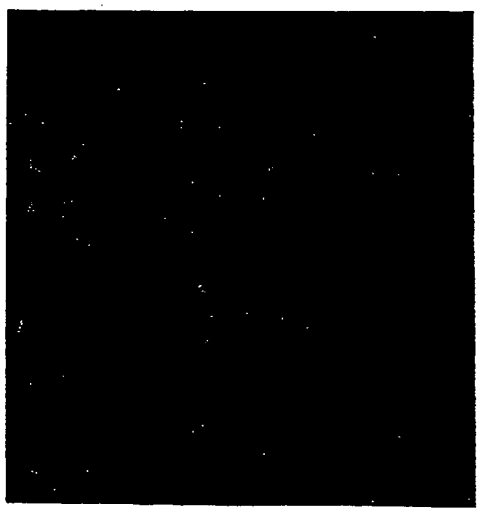


FIG. 17A.

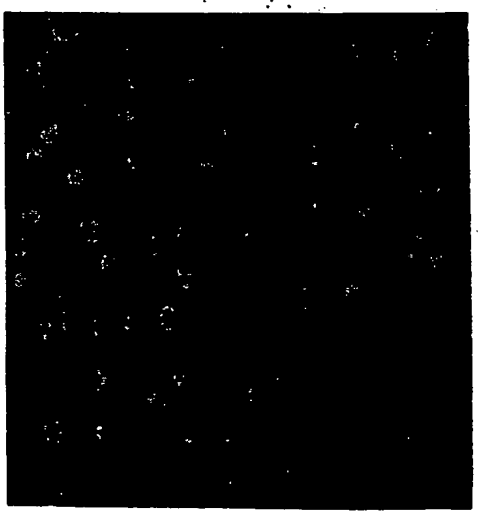


FIG. 17B.

GAATTCCAGCGCCGCCGTCATAGATCCACCCTGCCCAGGGGCTGTACATTGATCTCCGGCGTCAGTTCCTGGTAGGACAG
CACCGGCAGGGCGTAGAGATCGGCTTCGATCATCTTGCGCGTGTAGCGCCGGATGTCCATCGACGTCAGCAAGACGGGAC
GGCTCGCGCCGGCGGCCAGATCGCCGACACATTGGCGTATGTGCTCGACCAGCCGGCGTGTCGTGTCCGGATCGAGGGCG
AAATAACTGCCGGCGGCGGTCTGCCGGATGGCGGCGCGCACGGTTTCCTCGACCTTGGGGGCCAGCAGGTAGGCGGGCAG
GATATTGTGGCCGCTGGTGTACTTGTAGCTGATATAGCGCTTGAGTGCGATTCGGACATACTCCGTAAGCAGGACGGTAT
CCTTTTCCTTCTGGCCCCATTCGACCAGCGCTTCCAGGACGGCGCGCAGGTTGCGTATCGACACTTCCTCGGAAACAAGG
CGCTGCAGGATTTCGGCAATCTTCTGCACCGGCATGACGCGCAGGCACTCCTTGACCAGATCGGGAAATCGTTTTTCCAT
GGCCGAAAGCAGAAACCGGGTTTCCTGGATGCCGATGAAATCGGCTGAATATTTTTTCAGTACATATGCCAAGTGCCAAG
TCAGGATCTGGCTGATACCCAGGTAAGGAATACCTGCATCGCGCAAGGCGCCGGTCAGACTGGCTGCAACCCAGATCGTG
GGCGTATCGGGCAGAAAGGCCGCGCCCGTTTCGTATGCGATCCGCAGGGCCTGCAGGTTCTGCTCGGTGTCCCGCACCAG
CACGGCATCGTCGCGCAACATCCCCTGCGCCACCGGGATCTCCGACAGCACGATGGTGTAGGTATTGGCGGCCAGCGCTT
CGGTGAAGCGCAACTGGATGCCGGGAAATGGCACGCCCAAGTCGAAATAGAGCGCCCGCCGGATCTGCAGCAGATCGTCG
GTGAGGGTGGCCGGCTCGAACCGGGGCTGCAGCCGCGCGGCCACGTCGATGATCAGCGGGACGGTGGGAGCGAATTCCGC
CTGTCCATCCGCCGGCGCGCGGGTGCGGGGCAGGCCGTCGGCAGCCATGCCGGCGAGCGCGGGCCCGGCGCCTTCGGGCG
GACGCTGGGATGCGCGCAGCAGTACGAAACCGATGGTGCCCACCACGGCGGCCAGGGCGAAGAAGACCAGCGTGGGCATG
CCGGGAATGAGGCCCAGGCCTGCCGAGATCGCGCCGGCAATGACCAGGGCGCGAGGCTGCGCCAGCACTTGTGCGCCGAT
GTCGGTGCCTACGTTGGAGGGGCCATCCCCGGTCTGCACCCGCGTCACGATGATTCCCGCGCAGATGGCGATGAACAGCG
CCGGGATCTGCGCGATGAGCCCGTCGCCTATGGTCAGGATGGCATATGTCTGCACGGCCTCGCCGGCGCTCAGGCCGCGC
TGCAGCACGCCGACCAGCATGCCGCCAAGCAGGTTGACGGCAACGATGATCAGGCCGGCGATGGCATCGCCCTTGACGAA
CTTCATCGCGCCGTCCATGGCGCCATACAGTTGGCTTTCCTTCTCGACCGTACGGCGTCGGCGGCTTCGTCCATGT
CTATGGTGCCCGCGCGCAAGTCCGCGTCGATGGACATCTGCTTGCCGGGCATGGCGTCCAGCGAGAAGCGCGCGGCGACT
TCGGCCACCCGCTCCGCGCCTTTGGTGATGACCACGAACTGCACGATCGTGAGGATGAGGAAAACCACCAGGCCGACGAT
CAGGTTGCCGCCCACCACGAAGTTGCCGAAGGTCTCGATGATGTGGCCGGCATCGCCTTGCAGCAGGATCAGCCGCGTGG
TCGCGATGGAGATGCCCAGCCGGAACAGCGTGGTGACCAGCAGGACCGAAGGGAACGAGGAAAACGCCAGGGGCGAAGGC
AGGTACATCGCGACCATCAGCAGGACCGCGGACAGCGTCATGTTCGCACCGATCAGCACGTCGACCAGCGTCGGGTTGGG
CAACGGCAGGATCATCATGAAGACGATCGCCACGATGAGCACGGCCAGTACGATGTCGTTGCGGCTGGTGGCCAGCGCCA
CCGCGCGTTGCAGGCGGAGAATGGATTTCTTGCTCGTCATGGCGGGGGCGCGCTCGCGCAAGACGCCGCCCGTAGCGCCA
TATAGTCACGCATGGCCTGGCGGGCGTCGTCGGGTCGGTTCAGCGCCTGCATGGCCTGCGCCCGGACCAGATGGCCGGCG
GCATCGGGTGTGGCGCGCAGCGCGCGCTTGTCCAGCGTGACCAAGGCCATGCGCGGTTCGTCCTGGTGCAGATAGCCCAG
CGCCAGGGCCAGCAGGGAGCGGCTGTCGATCGCGTCCAGGGCATCCAGGGCCGCCAGCAGGGCAACCGTCTTGCTCCATT
GGCGCTGCAACTGGTATTGGTGCGCAAGCAATTGCAGAAGTTCGCGTACCTGAAGGCTGGGGTAGGGTAGGGTATGCGGC
ATCATCCCTGGTAGAGCGCGCTGCGATACATGGCAGCCAGGTCACGCAGCTTTCCGGCCTCGTTGAGGACGTGCAAGGCG
CGGCTCAAGGCCGGCGCGGTGTTGCCGGCGTGCAGTTCCATGGCCCGGGACAGTTCGTCGCGCGCCCGCGCCAGGGCGCG
CTCGAACTGCGCGGGCTGGAGCAATGCGCCATCCGTCAGGCGGGGCCGCGCCGACTCGTCGAGCATCGCGCTCAGGTCCG
GACGCACGAGCAGGGCTTTCAGATGATCGACCGCGCCGGTGGCGGGCGGCTCGAGCCAGCGCTCGGGTGGCAGGGTGGGG
GCGGGCTCGCAGCGGGGGCCGGGAACGATGTGGTCGACGCCTCGCTCCAGGCCAAGGTGCATGGGATGCATGCCGGGTAC
GGCGCTGGACATGGCGTCACGCCTCCAGGCGGTCGAGCCAGTTCGTCAGGCCCAGCACCGCCAGGCGCAACGCCGCTGCG
TCGAGCGAGCGTTCGGGCAGTCGTGTCTGCGCGACGATCCAGTCCTCGCTGTCCTCCGACCAGAGTGACGTCTGGATGGA
TCGGGGCGCCGCGGCGCTGTCCATGGGCCCGTTTCCATGCCGCCAGCAAGACGGAGGCGGCGTCGCGCTCCGCCCGCTGG
GCCAGGTGGACCAGGGCCGCGCCGGCGACACATTCGACGCCCAGGCGGCGCCCGTTGGACAGCGCCAGCGACGCCGATCG
GGACGGCCCGAATGCCAGGCCCTCGATGCCGATATCCTGGCCGAACTGATGCAGCGCCTTATCGGCAGTATTCATGCGTT
CTCCATTGCTATCGCGTTGTCCAGCGCATCCTGCGCGGCCGCCAGGACGGTGGCGCGCACGTCCATGTCGGCGTAGATCT
GCGTGGGCAGGTCTTTGAGAATCTGGCGTACGCCGCCGAGGAATGCGATGCGCTCGGAGAGGGCATTCGCGCCGTGGCGC
TCTGCCAGCTTCTCGAAGCGCGCGGGCGCAATCCATTTGTCCTCGCTGATTCCCACAATATCGCGCATCAGGCCCTGGGC
GTCCGCACACTCCTGCGAGCCTGCGTTGCCCAGCCGTTGTTTCAGGGCATTGCATTCCTCCAGTACCGTGGCGGCCACCT
CGACTTGATAGAGATCGCTCGCCAACACTTGCAGCCTGACGCCGTCCGTCGACGGTGTCGCCGCGGCCAGGTCGTGTCCC
AGCGCCTGAATCAGCGCGCCCAGCGCGCCGTGGATGTCGTCGTTCCCATAGCGTTCCAGCACCAGGTCCAGCGTGCGCGC
CAACGACAGCTGGCCCAGGGCGATGTCGCGGTACGCGTGCTGGAAGCCGGCCAGCTCGTCAGCGGAACGCGCGAACGCGC
CGGCCGTGGGCAGGGTGTTGATGCCGGCGCGGATTTCGGGGCCATGGGCGAGCTCCAGGTCGGCCAATGCATCGCGCAGG
GCTTCGAGCGCGTGCGGCGCGGCGTCCTCGTGCTCGCCGCGCTGCAGCGCGTGCTGCAGCGCGAGGTATTGCTGCGTGAC
ACCGGTAAACGCTTGCGCGGCCAGTTGCATGGGGGCGCCCCGGCCGCGCAGCAGCTCGGCGGTCAGGGCTTCCAGTTTTG
CCTGCGCGTCGGGGTCGTGGGTGTGGGAAAACAGTTCCGCAAGCTGCGCCGCGTCCAGCCAGAGCATCGGACGTTCGGCC
GTGACCTTGCGTTCGGAGTGATGCTTTTCCTCGGCAGCCTGCGCCATGTGCAGGCTGAGCTCCTCGGCCGCGTCCGCCAG
CGATATGCCGGTGGGCGCCGGTGCGATGCGCTGGCCTTGCAGCCAGCCGGAGGAAGTGTTGGCCGAGAAGGAACCATTTGCCT
CCTGCATGGCGGCGTGGAAGGGATTGGGGGCGGCATCGATACGAGTCATGGGGAGTCCTCGGAGGCGTCCTGGCGCC
ACTGGTGCAGTGAGTGTCGCGCGCGCGGCCATGGTTCCCGGAAACGGGCGCGATATTGGGCAATTCGCAGCCTGGAACTT
GCCGCGGGCGCAGGGTTACTCAGCATGCGTCTTTCAACTCGAAGGAGCCCTCATGACCATTGATCTCGGAGTTTCACTCA
CGTCGCAGGCCGGCGGCCTGCAAGGCATCGACCTCAAGAGCATGGATATCCAGACTCTCATGGTGTATGTGCAGGGTCGT
CGCGCCGAACTCCTCACGGCTCAAATGCAGACCCAGGCCGAAGTGGTGCAGAAGGCCAATGAACGCATGGCGCAGCTCAA
CGAGGTCCTGTCCGCGCTGTCCCGGGCCAAGGCCGAGTTTCCGCCCAATCCGAAGCCGGGCGACACCATCCCGGGCTGGG
ACAACCAGAAGGTCAGCCGGATCGAGGTTCCTCTCAATGATGCGCTGCGCGCTGCCGGCCTGACGGGCATGTTCGAAGCG
CGCGATGGCCAAGTGACCGCCCCCGGCGGCCGGGGTACGCAGGTCGTGAACGGCACGGGCGTCATGGCCGGTTCCACGAC

FIG. 18A

CTATAAGGAACTCGAAAGTGCCTACACCACCGTAAAGGGGATGCTGGATACGGCGTCCAATACGCAACAGATGGACATGA
TCAGGCTGCAGGCCGCCAGCAACAAGCGCAACGAGGCTTTCGAGGTCATGACCAACACCGAGAAGCGGCGCAGCGACCTG
AACAGTTCCATCACCAACAACATGCGCTAAGCGCTGCACAAAGGGGTATTCCATGCAGGAGCAAGACATCCAATCCATCA
TGCGTGCCGCGGAAGAGCTGGTCGAGCAGACCCGCAAGGCGTTGTGCAGCGTCGACGAGATCTACGCCCACGTTGGCGTC
GACCCCGCCCGCCTGCGCAACCTGGCGGTCGAGCAGGCAAGGATAGAGGCCGAGGCCCAGGCGGCGTTCCGTGATGACCT
CGCGGACATCGAGCGCGAGGCGGCGCGCGTCAAGGCGGCCAGCACCGATGCGCCGCAGGCCCGCAGGGTGCTTCACAACC
ACGTCTGAGCGCGGAGGCCTTCCATGCCAAAGTCAGCCGAGCAGGGCGGCTCCCCGGCGTCAGCTTCGCATGAGGCGTTG
CGCCATATTCTTGACGCAGGCGCTTCGATGGGCAGCTTGCAGGGGTTGGACGAGGTGCAACAGCAGGCGTTGTACGCGAT
CGCTCATGGCGCCTACGAACAGGGCCGCTATGCCGACGCGTTGAAAATGTTCTGCCTGCTGGTCGCGTGCGATCCGCTGG
AAGCCCGTTATCTGCTGGCCCTGGGCGCCGCGGCCCAGGAGCTGGGGCTGTACGAGCATGCCTTGCAGCAATACGCGGCC
GCGGCGGCTTTGCAAGTTGGATTCCCCCCAGGCCCCTGTTGCATGGCGCCGAGTGCCTGTATGCGTTGGTTCGTCGCCGC
GACGCCCTGGATACGTTCGACATGGTGCTTGAGTTGTGCGGGTCGCCGGAGCATGCGGCCCTGCGCGAACGGGCCGAGTC
GCTGCGCAGGAGCTATGCACGTGCCGACTGAAACGGCGCCATGTCCGCCGTCAAGATTTCAATTCGAGGAGGTTAGATAT
GTCTGTTTCTCCGACTTCGCCCGGCTCTTTCGGGGCCGGCCCTGTCTTTGACTCCGAATTGCAGGCCCCGGCCCCGTCGG
CGCAGCGTCGCGGCGGTGCGGCGCCTGTGCCGCCGCCCGTCGATCGGCGCGGAGTCGAGCCGGGAGATCCCACGCTGGGC
ATGCTGCCCGCGCCCGATTTGCTCGCGGGGGGCGCCGTCAGCCGCACCCGCGCGGCGCTCGACGATCTGGACGCAGCACG
GCTCGGTGAAGACATCTACACCTTGATGGCGGTGTTGCAACAGGCCAGTCAACAGATGCGGGAGGCCGCCCGTATCGCTC
GTGATGCCGAGGCCACGCGGCAAACGCAGGCTATCGGCGATGCGGCCAGCCAGATGCGCCAGGCGGCCAACGAGCGCATG
GCCGGAGCGATCGTGGCGGGCGCCATGCAGATAGCGGGTGGTTTCGTGCAGCTGGGGGCGGGCCTGGCAGCGGGTTTGCA
GGCCATGGGTGGCGCAGCTGCGCAAGCCAAGGGCGCCGCATTTTCCGAGCAGGCCTCGACAAGCCGCAAGGTGGCGGCCG
GCTTGCACGATGCCCCCGAGCTGCAGGCAACGGTGCAGGCCCGCGCAACCCAGCTCGAAGCGCAAGCGGCTTCGTTTGGT
GCTGACGCGGCTCGTTCGTCGGCAAAGTCGCAGCGCGTATCGAGCGTTGCCCAGGCCGGCGCCGCGACGGCCGGCGGTAT
CGGCGGCCTGACCAGCGCCGCCCAGGAACGCCGCGCCGCCGAGCACGAGGCCAGGCGCGCGGAGCTGGACGTCGAAGCGA
AGGTGCATGAAACGGCCTCGCGGCGGGCCGACGAAGCCATGCAGCAGATGCTCGACATCATCCGCGGCATCAGGGAAAAG
CTGGCCGGGATGGAGCAGTCCCGCAGCGAGACCGCCCGTAGCGTGGCCCGCAATATCTGAGTGTCCGGCTCCAACCTTCA
ATCTTGAGGATGACCGTCATGAGTACGACCATATCCACAGCCCCGAGCGGCGCCGCGCTTGCGCCGTCTCGCATAGATAT
GCGGGCACCGGAGCCCGGGAGTGCCGGCGAAGGCGCCGGCATCCTGGCGCCGGTGACGACGCTGGCTCTGGCGGCGGCC
GGCCGGCTTTTCCAGCGTCACCGTCGCTGCGCACCGCGCCCGTCCTGGATCCGCCAGTGCGCGATCTCAGCCCCGCCGAC
TTGGCCGACCTGCTGCGCGTCTTGCGATCCAGGGCGGTGGACGGGCAGTTGGCCACGGCGCGCGAGAACCTGCAGGACGC
GCAAGTCAAGGCGAAGCAGAACACCCAGGCCCAGCTCGACAAGCTGGACGCATGGTTTCGGAAGGCCGAAGAGGCCGAGA
GCAAGGGATGGCTGAGCAAGGTGTTCGGCTGGATCGGCAAGGTGCTGGCGGTCGTGGCATCGGCCCTGGCGGTGGGCTTT
GCCGCCGTCGCCAGCGTGGCCACCGGCGCGGCGGCCACACCCATGCTGCTGCTCAGCGGCATGGCACTGGTCAGCGCCGT
GACATCGCTGGCCGACCAGATATCGCAAGAAGCGGGAGGCCCGCCTATCAGCCTGGGCGGGTTTCTCTCCGGGCTGGCCG
GACGTCTGCTGACAGCGTTGGGGGTGGATCAGTCGCAGGCCGACCAAATTGCCAAGATCGTCGCCGGCCTGGCCGTGCCC
GTCGTCTTGCTGATCGAACCCCAGATGCTGGGCGAAATGGCGCAAGGCGTGGCCAGGCTGGCTGGCGCCAGCGATGCCAC
CGCGGGGTACATAGCCATGGCGATGTCCATCGTGGCGGCGATCGCGGTCGCCGCGATCAATGCCGCCGGTACAGCCGGCG
CGGGTAGCGCTTCGGCGATCAAGGGGGCCTGGGATCGGGCCGCCGCGGTAGCCACCCAGGTCCTTCAAGGGGGTACGGCA
GTGGCGCAAGGCGGCGTCGGCGTGTCGATGGCAGTCGATCGCAAACAGGCCGATTTCTTGCTCGCCGACAAGGCGGATCT
GGCGGCGAGCCTGACAAAACTGCGGGCGGCCATGGAGCGTGAGGCGGACGATATCAAGAAGATCCTGGCTCAATTCGACG
AGGCCTATCACATGATCGCGAAGATGATCAGCGATATGGCGAGTACGCACAGCCAGGTCAGCGCCAACCTCGGGCGGCGC
CAGGCGGTGTAGCGCCGGGCGCTCAAGGAATTTTCATGACTGTTCACGACGACGCGGCCGCGGCGCTGCGCGCCCGGCTG
GATGCGCTGCCGGGCAGCCGGCGCCTGACGGCCGAGCAATTGGAAGTGATTTACGCGATGGCGTATGCGCACGTCGCCAG
GTGCGAGTACGGCAAGGCGCTGCCCATCTTCGCCTTCCTCGCGCAGTATGGTCCCACGCGCAAGCATTACTGGGCCGGGC
TGGCGCTATGCCTGCAGAAGACCGACCGTCCCGACGAGGCGCGCAATATCTATGCGTTGATCCTCACGTTCTATCCCGAT
TCCGCGGACGCCGTGTTGCGCACGGCCGAATGCGAGCTGGCGTTGGGTGAGAACGAACGGGCACAGGCGGCCCTGTTCGG
CGCAATTGCCATCGATGCAGAAAGTGGGCAGCCAGGTCCGGTCTCGCACCGTGCGCGCGCTTTGCTCGATCTTATTTCAG
TTTCACATCCGGAGTAACTCCATGCATTCAGACTCAGGTTCAGATTCAGGCTCAGACTCAGGCTCAGGCTCACCCATGGC
CTCGTCGATACATCCATCGGAACCGATACAGCCGATGGAGCATGTGCTCGAGGAGGCCGACGCCCGCCTGCTTACCGAAG
TGGGCTTTCTGGCGGCGGCCGTCAGCGATCTGACGCGCGCGGACGCCATTTTCAATGCATTGCAACGTGTACGGCCGGGC
CGGACGTATCCCTGCATCGGCCTGGCGGTCGCCCGCATGAACGCCGGGCTGCCCGACGAAGCCGCCGAGATCCTGGCGAA
TTTCCAGCCGGCACAGGCGGAGGACCGCTCGGAACTGGACGCCTGGTGCGGGTTCGCCCTGTTGCTGGCCGGCCGCTCGG
ACGAGGCGCGCCGCATGCTGCAGCGAGCCATCGATGCGGGTGGCGAGGCGGCAAGGCTGGCGCAGGTCGTGTTGGACAGC
GGACCCGCCATGATGCGGCCCGCGCCGTTGCAGTCCGAGCCATTACCTGGAGCTCCTGGATGAATTTGGATCTGACGGCG
ATCAACGCCGTGCAGGAACGGCTGCTCGCTCGATCGTTCGACATGCCGCGGTCTCCCGCGATGGCGGATCAGGCGCGCTT
TGAGTTGGCGCTGGGCGAGATGCCCGGCGCATCGGCCCCGAACGGGGCGATCGCCCCGGCACCGGCCGAAGGCCCGGCGG
CGCCGGTCGCGCTCGGCGAGCCGCTGGGCCGCCGCATTCTTGGACAGTTGCGCGGCGGCCTGGCCGATGTGGCAGGAAAA
TGGCGGGCGGTGCAGACGGGCTTGGCCGAGGTGAGCCAGGCGCCTACCGTGGTGGGTATGCTCGATCTGCAGGCCAGGTT
GCTACAGGCATCCGTGGAGTACGAGTTGGTGGGCAAGGCAATAGGGCGCGCCACCCATAACGTCTACACGCTGGCGAGAA
TGTCATGAACGCCATCGGGGCGATCCAACGGTATCGGCGCGGCGCGGGATGGGCGGCCCTGGCGCTCGCCCTGGCGCTGC
TGGCCGGCTGCGGCGCCCGCGTCGAGCTGTTGGGCGCGGCGCCCGAGAACGAAGCCAACGAAGTATTGGCGGCGCTGCTC
GAGGCAGGCATCGCTGCGCAGAAGCAGTCCGGCAAGGCCGGCTACGCGGTTTCGGTGCCGGCCGAGGCGGTGGCCCGGTC
GCTGGAGATCCTGCGCGCAAGCGGCCTGCCCCGCGAGCAGTTCGACGGAATGGGGCGCATATTCCGCAAGGAAGGCCTGG

FIG. 18B

TTTCATCGCCGCTCGAAGAGCGCGCCCGCTACATTTATGCGCTATCTCAGGAATTGGCCGACACCCTGTCGCAGATCGAC
GGCGTGCTCAGCGCCCGCGTGCACGTGGTGCTTCCCGAGCGCGGCGCGGTCGGCGAGCCGGCCACCCCTTCGACGGCAGG
GGTGTTTCTCAAGTACCGCGACGGACAGAGCCTCGACGCGCTCGTGCCCGAGATCCGCAAGCTGGTCACGCATGCCATCC
CGGGCCTGGCCGAGGACCGTGTATCGGTTGCCCTGGTGGTGGCCCAGCCCGTTCAGGCCGCACCCGTGCCGGTCGCGTGG
CGCCGCGTGCTTGGCGTACAAGTCGCGGACGGATCGGTCCTGAGATTTTCGCTGTTGCTGCTGTTGTTGCCGGTGCTGTG
CCTGATAGTGGCGGGGGCCACGCTCTACGCCTGGCGCACGCGCTGGTCCCGAGCGGCTCATGATATTCAATCTCCTGCCCAG
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CCTGACCCTGCATGCCAGTCGCCACGACGAGACGTTTCCAGCCGATTGGGTGCGCGCGTTGTGCAATGCCGACGCGGCGT
TGGCCAACGCATGGCATCGCCATTGGTCGCGCTGGATCTTGTGCGAGCTGGGCCTGCTGAACCAGCCGGTCCTGAGCCTC
GATCCGCCGCAGTTGAAGGTCGCGCTATTGTCCACGGACGCCTTGCGCACCTGCGCCGCGCTCGGGCGCGATGTGATGAATT
CGCGCCGCGCCTGCGACGCGCGATAGACGGCGCTGAGGTCCGTACCTTGCATGCCGCGCTCGGACCCTGGCCGCCACGGTCCAG
TCGCCGTGTCTTCCGCGGCGCGGGCCCTGCATGACGGGATCGCCGCCAGTTCGGACTGGACCCTGGCCGCCACGGTCCAG
GCGGCGCAGAAACTGGGCTGGGCCGTGCTGCGCGACGCCGTGCAGGGCGCCGCCGACGAGATAGCGCTGCGTTGCGCGCT
GAAGTTGCCGCGCGACCTTGATCCCGCGCCCGTCCTGCCGCCCGAGGCGGCGCTTGCGCTGGTGCTGTCCATGCTCGAAA
TCCTGGATGCCGAATGGCTTTCCTCGTTCCCCGCCCAAGCCTGATCCAGGCGGTACGGCCCGGCCGTGCGGATCCCGCGA
CCGACGTCTTGCGCGCCGAAGACTACGCCGAGCTGCTCAGCGCCGCGCAGATCGTTGCCCAGGCACATCGGCGGGCCGAC
GAAATCGTGGCCGAGGCGCGAGAGGAGTTCGAGCGCGAGCGCAGGCGAGGCTATGAGGAGGGGCGCCGCGAAGCGCTTAC
GGATCAGGCGGAGAAGATGATAGAAACGGTAAGCCGCACGATCGACTACTTCGCGGGTATCGAGAACGAGATGATCGAAC
TGGTCATGAGCGCGGTCCGCAAGAACGTCGACGGTTACGACGACCGCGAGCGCACCGTGATCGCCGTGCGCAACGCATTG
GCGGTCGTGCGCAATCAGCGCCAGATGACCTTGCGCCTGCACCCCGACGAGGTGGATGTGCTCCGGGAAGGCATGAACCA
GCTGCTGGCGGCCTATCCGGGCGTGGGCTACCTGGACCTGCTGCCCGACGCCAGGCTGACGCCGGGAGCCTGCATACTGG
AGAGCGAGATAGGCATGGTCGAGGCCAGCCTCGAGGACCAGCTGTGCGCCTTGCGGGCGGCCTTCGAACGTACATTCGGC
CGGCGCGGATAGGGGCATGCGTCAGTACCACTACATCACGGAGATGATGCGGGTGGCCCTGCAGGATCTGTCCACGTTGC
GGATAAAGGGCCGAGTGGTGCAAGTGGTGGGAACGATCATCAAGGCCGTCGTTCCGATGGTCAAGATCGGCGAAGTGTGC
CTGCTGCGCAATCCTGGCGAGGACTTCGAGATGCACGGCGAAGTGGTGGGCTTTGTTCGCGACGCCGCCTTGCTCACGCC
CATCGGCGACATGTACGGGATTTCCTCGGCGACCGAGGTGATACCGACCGGACGCACGCATATGGTGCCCGTCGGTCCGG
GCTTGCTGGGACGCGTGCTGGACGGGCTGGGACGTCCGCTGGACGTCGCCGAGTCAGGGCCGCTGCATGCCCACAAGTTC
TATCCGGTCTTCGCCGATGCGCCGGATCCGCTGACGCGTCGCATCATCCATGCTCCGCTGGAGCTGGGGGTGCGCGTACT
GGACGGTTTGCTTACATGCGGGGAAGGCCAGCGTCTGGGAATTTTCGCAGCAGCCGGCGGCGGCAAGTCGACCTTGCTGG
GCATGCTGGTCAAGGGCGCCGCGGTCGACGTGACGGTGGTGGCGCTGATCGGCGAGCGTGGGCGGGAAGTTCGCGAGTTC
CTTGAGCACGAACTCGGTCCGGAGGGCAGACGCAAGAGCGTGATCGTTTGCGCGACCAGCGACAAGTCCTCGATGGAGCG
TGCCAAGGCGGCATACGTCGCAACCGCCATCGCCGAATACTTCCGCGATCAAGGGCAGCGTGTACTTTTTCGGATGGATT
CGGTCACCCGCTTTGCGCGAGCCCAGCGTGAAATCGGCTTGGCGGCAGGCGACCCGCCGACGCGGCGCGGCTATCCGCCG
TCGGTGTTCGCCACCCTGCCAAAACTGATGGAGCGCGCCGGCATGAACCAGACGGGTTCGATCACGGCGCTGTATACGGT
GCTGGTCGAGGGGGACGACATGAACGAACCGGTGGCCGACGAGACGCGTTCGATACTGGACGGCCACATCGTGCTCTCGC
GCAAGCTGGGAGCGGCGAATCACTATCCTGCCGTCGACGTCCTGGCCTCAGCCAGCCGGGTCATGAATGCCGTGGTGTCG
CCACGTCACAAGTACCTGGCCGGACGTATGCGCGAACTGATGGCCAAGTACCAGGACGTCGAGCTGTTGGTGAAAATCGG
CGAGTACAAGCAGGGCGCCGATGCGTCGACCGATGAGGCGATACAGAAGATCGGACAGATCAATGCGTTTCTCAGACAAC
TAACCGACGAACGCGAAGCATTCGAGGATACCGTACTGCGCATGGCTGAAATCATCGGACCCGAATCCTAATGGACCTGG
AAAGCCTGCTTGCCATCAAGCATTTTCGCGCCGACCAAGCCCAGCTTGCGCTGAAACGCCAACAGCAGGCCTGCGCGGTT
GCTGCCGCGGCGCAGCGCCAGGCGCAAGGCCGCCTCGACAATTGTCGCCTGTGGGCCGGGCAGCTCGAAAACCGTCTTTA
TGCCGAGCTGTGCCGGCGCATCGTCAAGACACGCGACATCGACGAGGTGCTGCAACGAGTGGGCCACGCCCGCGACCGCC
AGGCCAGCCTGGCGCTGCAGCTCGACAACGCCGTGCGCCGCCACGAGCATGAAATCCAGCTGCTCGCGCAGCAGCGCGAG
CAGCACCGGGAGTGCTTCCAGGCGCAGCAACGGATCGCCGAGTTGGTGCGCCTGCAGCAGGTCGAGGCGGCGGCCTTGCG
CGAGAGCCTGGAGGATCTCTAAATTCAGGAAGCCATCGAATTGTCGGCGCGTGGGCGCGA

FIG. 18C

BSCV

ATGACGAGCAAGAAATCCATTCTCCGCCTGCAACGCGCGGTGGCGCTGGCCACCAGCCGCAACGACATCGTACTGGCCGT
GCTCATCGTGGCGATCGTCTTCATGATGATCCTGCCGTTGCCCAACCCGACGCTGGTCGACGTGCTGATCGGTGCGAACA
TGACGCTGTCCGCGGTCCTGCTGATGGTCGCGATGTACCTGCCTTCGCCCCTGGCGTTTTCCTCGTTCCCTTCGGTCCTG
CTGGTCACCACGCTGTTCCGGCTGGGCATCTCCATCGCGACCACGCGGCTGATCCTGCTGCAAGGCGATGCCGGCCACAT
CATCGAGACCTTCGGCAACTTCGTGGTGGGCGGCAACCTGATCGTCGGCCTGGTGGTTTTCCTCATCCTCACGATCGTGC
AGTTCGTGGTCATCACCAAAGGCGCGGAGCGGGTGGCCGAAGTCGCCGCGCGCTTCTCGCTGGACGCCATGCCCGGCAAG
CAGATGTCCATCGACGCGGACTTGCGCGCGGGCACCATAGACATGGACGAAGCCCGCCGCCGACGCCGTACGGTCGAGAA
GGAAAGCCAACTGTATGGCGCCATGGACGGCGCGATGAAGTTCGTCAAGGGCGATGCCATCGCCGGCCTGATCATCGTTG
CCGTCAACCTGCTTGGCGGCATGCTGGTCGGCGTGCTGCAGCGCGGCCTGAGCGCCGGCGAGGCCGTGCAGACATATGCC
ATCCTGACCATAGGCGACGGGCTCATCGCGCAGATCCCGGCGCTGTTCATCGCCATCTGCGCGGGAATCATCGTGACGCG
GGTGCAGACCGGGGATGGCCCCTCCAACGTAGGCACCGACATCGGCGCACAAGTGCTGGCGCAGCCTCGCGCCCTGGTCA
TTGCCGGCGCGATCTCGGCAGGCCTGGGCCTCATTCCCGGCATGCCCACGCTGGTCTTCTTCGCCCTGGCCGCCGTGGTG
GGCACCATCGGTTTCGTACTGCTGCGCGCATCCCAGCGTCCGCCCGAAGGCGCCGGGCCCGCGCTCGCCGGCATGGCTGC
CGACGGCCTGCCCCGCACCCGCGCGCCGGCGGATGGACAGGCGGAATTCGCTCCCACCGTCCCGCTGATCATCGACGTGG
CCGCGCGGCTGCAGCCCCGGTTCGAGCCGGCCACCCTCACCGACGATCTGCTGCAGATCCGGCGGGCGCTCTATTTCGAC
TTGGGCGTGCCATTTCCCGGCATCCAGTTGCGCTTCACCGAAGCGCTGGCCGCCAATACCTACACCATCGTGCTGTCGGA
GATCCCGGTGGCGCAGGGGATGTTGCGCGACGATGCCGTGCTGGTGCGGGACACCGAGCAGAACCTGCAGGCCCTGCGGA
TCGCATACGAAACGGGCGCGGCCTTTCTGCCCGATACGCCCACGATCTGGGTTGCAGCCAGTCTGACCGGCGCCTTGCGC
GATGCAGGTATTCCTTACCTGGGTATCAGCCAGATCCTGACTTGGCACTTGGCATATGTACTGAAAAAATATTCAGCCGA
TTTCATCGGCATCCAGGAAACCCGGTTTCTGCTTTCGGCCATGGAAAAACGATTTCCCGATCTGGTCAAGGAGTGCCTGC
GCGTCATGCCGGTGCAGAAGATTGCCGAAATCCTGCAGCGCCTTGTTTCCGAGGAAGTGTCGATACGCAACCTGCGCGCC
GTCCTGGAAGCGCTGGTCGAATGGGGCCAGAAGGAAAAGGATACCGTCCTGCTTACGGAGTATGTCCGAATCGCACTCAA
GCGCTATATCAGCTACAAGTACACCAGCGGCCACAATATCCTGCCCGCCTACCTGCTGGCCCCCAAGGTCGAGGAAACCG
TGCGCGCCGCCATCCGGCAGACCGCCGCCGGCAGTTATTTCGCCCTCGATCCGGACACGACACGCCGGCTGGTCGAGCAC
ATACGCCAATGTGTCGGCGATCTGGCCGCCGGCGCGAGCCGTCCCGTCTTGCTGACGTCGATGGACATCCGGCGCTACAC
GCGCAAGATGATCGAAGCCGATCTCTACGCCCTGCCGGTGCTGTCCTACCAGGAACTGACGCCGGAGATCAATGTACAGC
CCCTGGGCAGGGTGGATCTATGA

FIG. 19

BCR3/BSCX

ATGTCCAGCGCCGTACCCGGCATGCATCCCATGCACCTTGGCCTGGAGCGAGGCGTCGACCACATCGTTCCCGGCCCCCG
CTGCGAGCCCGCCCCCACCCTGCCACCCGAGCGCTGGCTCGAGCCGCCCGCCACCGGCGCGGTCGATCATCTGAAAGCCC
TGCTCGTGCGTCCGGACCTGAGCGCGATGCTCGACGAGTCGGCGCGGCCCCGCCTGACGGATGGCGCATTGCTCCAGCCC
GCGCAGTTCGAGCGCGCCCTGGCGCGGGCGCGCGACGAACTGTCCCGGGCCATGGAACTGCACGCCGGCAACACCGCGCC
GGCCTTGAGCCGCGCCTTGCACGTCCTCAACGAGGCCGGAAAGCTGCGTGACCTGGCTGCCATGTATCGCAGCGCGCTCT
ACCAGGGATGA

FIG. 20

BOPN

ATGACTCGTATCGATGCCGCCCCCAATCCCTTCCACGCCGCCATGCAGGGGCGCCAGGACGCCTCGGCCAACACTTCCTC
CGGCTGGCTGCAAGGCCAGCGCATCGCACCGGCGCCCACCGGCATATCGCTGGCGGACGCGGCCGAGGAGCTCAGCCTGC
ACATGGCGCAGGCTGCCGAGGAAAAGCATCACTCCGAACGCAAGGTCACGGCCGAACGTCCGATGCTCTGGCTGGACGCG
GCGCAGCTTGCGGAACTGTTTTCCCACACCCACGACCCCGACGCGCAGGCAAAACTGGAAGCCCTGACCGCCGAGCTGCT
GCGCGGCCGGGGCGCCCCCATGCAACTGGCCGCGCAAGCGTTTACCGGTGTCACGCAGCAATACCTCGCGCTGCAGCACG
CGCTGCAGCGCGGCGAGCACGAGGACGCCGCGCCGCACGCGCTCGAAGCCCTGCGCGATGCATTGGCCGACCTGGAGCTC
GCCCATGGCCCCGAAATCCGCGCCGGCATCAACACCCTGCCCACGGCeGGCGCGTTCGCGCGTTCCGCTGACGAGCTGGC
CGGCTTCCAGCACGCGTACCGCGACATCGCCCTGGGCCAGCTGTCGTTGGCGCGCACGCTGGACCTGGTGCTGGAACGCT
ATGGGAACGACGACATCCACGGCGCGCTGGGCGCGCTGATTCAGGCGCTGGGACACGACCTGGCCGCGGCGACACCGTCG
ACGGACGGCGTCAGGCTGCAAGTGTTGGCGAGCGATCTCTATCAAGTCGAGGTGGCCGCCACGGTACTGGAGGAATGCAA
TGCCCTGAAACAACGGCTGGGCAACGCAGGCTCGCAGGAGTGTGCGGACGCCCAGGGCCTGATGCGCGATATTGTGGGAA
TCAGCGAGGACAAATGGATTGCGCCCGCGCGCTTCGAGAAGCTGGCAGAGCGCCACGGCGCGAATGCCCTCTCCGAGCGC
ATCGCATTCCTCGGCGGCGTACGCCAGATTCTCAAAGACCTGCCCACGCAGATCTACGCCGACATGGACGTGCGCGCCAC
CGTCCTGGCGGCCGCGCAGGATGCGCTGGACAACGCGATAGCAATGGAGAACGCATGA

FIG. 21

BSP22

ATGACCATTGATCTCGGAGTTTCACTCACGTCGCAGGCCGGCGGCCTGCAAGGCATCGACCTCAAGAGCATGGATATCCA
GACTCTCATGGTGTATGTGCAGGGTCGTCGCGCCGAACTCCTCACGGCTCAAATGCAGACCCAGGCCGAAGTGGTGCAGA
AGGCCAATGAACGCATGGCGCAGCTCAACGAGGTCCTGTCCGCGCTGTCCCGGGCCAAGGCCGAGTTTCCGCCCAATCCG
AAGCCGGGCGACACCATCCCGGGCTGGGACAACCAGAAGGTCAGCCGGATCGAGGTTCCTCTCAATGATGCGCTGCGCGC
TGCCGGCCTGACGGGCATGTTCGAAGCGCGCGATGGCCAAGTGACCGCCCCCGGCGGCCGGGGTACGCAGGTCGTGAACG
GCACGGGCGTCATGGCCGGTTCCACGACCTATAAGGAACTCGAAAGTGCCTACACCACCGTAAAGGGGATGCTGGATACG
GCGTCCAATACGCAACAGATGGACATGATCAGGCTGCAGGCCGCCAGCAACAAGCGCAACGAGGCTTTCGAGGTCATGAC
CAACACCGAGAAGCGGCGCAGCGACCTGAACAGTTCCATCACCAACAACATGCGCTAA

FIG. 22

BCRH1

ATGCCAAAGTCAGCCGAGCAGGGCGGCTCCCCGGCGTCAGCTTCGCATGAGGCGTTGCGCCATATTCTTGACGCAGGCGC
TTCGATGGGCAGCTTGCAGGGGTTGGACGAGGTGCAACAGCAGGCGTTGTACGCGATCGCTCATGGCGCCTACGAACAGG
GCCGCTATGCCGACGCGTTGAAAATGTTCTGCCTGCTGGTCGCGTGCGATCCGCTGGAAGCCCGTTATCTGCTGGCCCTG
GGCGCCGCGGCCCAGGAGCTGGGGCTGTACGAGCATGCCTTGCAGCAATACGCGGCCGCGGCGGCTTTGCAAGTTGGATT
CCCCCCAGGCCCCTGTTGCATGGCGCCGAGTGCCTGTATGCGTTGGTTCGTCGCCGCGACGCCCTGGATACGTTCGACAT
GGTGCTTGAGTTGTGCGGGTCGCCGGAGCATGCGGCCCTGCGCGAACGGGCCGAGTCGCTGCGCAGGAGCTATGCACGTG
CCGACTGAAACGGCGCCATGTCCGCCGTCAAGATTTCAATTCGAGGAGGTTAGATATGTCTGTTTCTCCGACTTCGCCCG
GCTCTTTCGGGGCCGGCCCTGTCTTTGA

FIG. 23

BOPD

ATGTCTGTTTCTCCGACTTCGCCCGGCTCTTTCGGGGCCGGCCCTGTCTTTGACTCCGAATTGCAGGCCCCGGCCCCGTC
GGCGCAGCGTCGCGGCGGTGCGGCGCCTGTGCCGCCGCCCGTCGATCGGCGCGGAGTCGAGCCGGGAGATCCCACGCTGG
GCATGCTGCCCGCGCCCGATTTGCTCGCGGGGGGCGCCGTCAGCCGCACCCGCGCGGCGCTCGACGATCTGGACGCAGCA
CGGCTCGGTGAAGACATCTACACCTTGATGGCGGTGTTGCAACAGGCCAGTCAACAGATGCGGGAGGCCGCCCGTATCGC
TCGTGATGCCGAGGCCACGCGGCAAACGCAGGCTATCGGCGATGCGGCCAGCCAGATGCGCCAGGCGGCCAACGAGCGCA
TGGCCGGAGCGATCGTGGCGGGCGCCATGCAGATAGCGGGTGGTTTCGTGCAGCTGGGGGCGGGCCTGGCAGCGGGTTTG
CAGGCCATGGGTGGCGCAGCTGCGCAAGCCAAGGGCGCCGCATTTTCCGAGCAGGCCTCGACAAGCCGCAAGGTGGCGGC
CGGCTTGCACGATGCCCCCGAGCTGCAGGCAACGGTGCAGGCCCGCGCAACCCAGCTCGAAGCGCAAGCGGCTTCGTTTG
GTGCTGACGCGGCTCGTTCGTCGGCAAAGTCGCAGCGCGTATCGAGCGTTGCCCAGGCCGGCGCCGCGACGGCCGGCGGT
ATCGGCGGCCTGACCAGCGCCGCCCAGGAACGCCGCGCCGCCGAGCACGAGGCCAGGCGCGCGGAGCTGGACGTCGAAGC
GAAGGTGCATGAAACGGCCTCGCGGCGGGCCGACGAAGCCATGCAGCAGATGCTCGACATCATCCGCGGCATCAGGGAAA
AGCTGGCCGGGATGGAGCAGTCCCGCAGCGAGACCGCCCGTAGCGTGGCCCGCAATATCTGA

FIG. 24

BOPB

ATGACCGTCATGAGTACGACCATATCCACAGCCCCGAGCGGCGCCGCGCTTGCGCCGTCTCGCATAGATATGCGGGCACC
GGAGCCCGGGAGTGCCGGCGAAGGCGCCGGCATCCTGGCGCCGGTGACGACGCTGGCTCTGGCGGCGGGCCGGCCGGCTT
TTCCAGCGTCACCGTCGCTGCGCACCGCGCCCGTCCTGGATCCGCCAGTGCGCGATCTCAGCCCCGCCGACTTGGCCGAC
CTGCTGCGCGTCTTGCGATCCAGGGCGGTGGACGGGCAGTTGGCCACGGCGCGCGAGAACCTGCAGGACGCGCAAGTCAA
GGCGAAGCAGAACACCCAGGCCCAGCTCGACAAGCTGGACGCATGGTTTCGGAAGGCCGAAGAGGCCGAGAGCAAGGGAT
GGCTGAGCAAGGTGTTCGGCTGGATCGGCAAGGTGCTGGCGGTCGTGGCATCGGCCCTGGCGGTGGGCTTTGCCGCCGTC
GCCAGCGTGGCCACCGGCGCGGCGGCCACACCCATGCTGCTGCTCAGCGGCATGGCACTGGTCAGCGCCGTGACATCGCT
GGCCGACCAGATATCGCAAGAAGCGGGAGGCCCGCCTATCAGCCTGGGCGGGTTTCTCTCCGGGCTGGCCGGACGTCTGC
TGACAGCGTTGGGGGTGGATCAGTCGCAGGCCGACCAAATTGCCAAGATCGTCGCCGGCCTGGCCGTGCCCGTCGTCTTG
CTGATCGAACCCCAGATGCTGGGCGAAATGGCGCAAGGCGTGGCCAGGCTGGCTGGCGCCAGCGATGCCACCGCGGGGTA
CATAGCCATGGCGATGTCCATCGTGGCGGCGATCGCGGTCGCCGCGATCAATGCCGCCGGTACAGCCGGCGCGGGTAGCG
CTTCGGCGATCAAGGGGGCCTGGGATCGGGCCGCCGCGGTAGCCACCCAGGTCCTTCAAGGGGGTACGGCAGTGGCGCAA
GGCGGCGTCGGCGTGTCGATGGCAGTCGATCGCAAACAGGCCGATTTCTTGCTCGCCGACAAGGCGGATCTGGCGGCGAG
CCTGACAAAACTGCGGGCGGCCATGGAGCGTGAGGCGGACGATATCAAGAAGATCCTGGCTCAATTCGACGAGGCCTATC
ACATGATCGCGAAGATGATCAGCGATATGGCGAGTACGCACAGCCAGGTCAGCGCCAACCTCGGGCGGCGCCAGGCGGTG
TAG

FIG. 25

BCRH2

ATGACTGTTCACGACGACGCGGCCGCGGCGCTGCGCGCCCGGCTGGATGCGCTGCCGGGCAGCCGGCGCCTGACGGCCGA
GCAATTGGAAGTGATTTACGCGATGGCGTATGCGCACGTCGCCAGGTGCGAGTACGGCAAGGCGCTGCCCATCTTCGCCT
TCCTCGCGCAGTATGGTCCCACGCGCAAGCATTACTGGGCCGGGCTGGCGCTATGCCTGCAGAAGACCGACCGTCCCGAC
GAGGCGCGCAATATCTATGCGTTGATCCTCACGTTCTATCCCGATTCCGCGGACGCCGTGTTGCGCACGGCCGAATGCGA
GCTGGCGTTGGGTGAGAACGAACGGGCACAGGCGGCCCTGTTCGGCGCAATTGCCATCGATGCAGAAAGTGGGCAGCCAG
GTCCGGTCTCGCACCGTGCGCGCGCTTTGCTCGATCTTATTTCAGTTTCACATCCGGAGTAA

FIG. 26

BCR4/BSCY

ATGGAGCATGTGCTCGAGGAGGCCGACGCCCGCCTGCTTACCGAAGTGGGCTTTCTGGCGGCGGCCGTCAGCGATCTGAC
GCGCGCGGACGCCATTTTCAATGCATTGCAACGTGTACGGCCGGGCCGGACGTATCCCTGCATCGGCCTGGCGGTCGCCC
GCATGAACGCCGGGCTGCCCGACGAAGCCGCCGAGATCCTGGCGAATTTCCAGCCGGCACAGGCGGAGGACCGCTCGGAA
CTGGACGCCTGGTGCGGGTTCGCCCTGTTGCTGGCCGGCCGCTCGGACGAGGCGCGCCGCATGCTGCAGCGAGCCATCGA
TGCGGGTGGCGAGGCGGCAAGGCTGGCGCAGGTCGTGTTGGACAGCGGACCCGCCATGATGCGGCCCGCGCCGTTGCAGT
CCGAGCCATTACCTGGAGCTCCTGGATGA

FIG. 27

BSCI

ATGAATTTGGATCTGACGGCGATCAACGCCGTGCAGGAACGGCTGCTCGCTCGATCGTTCGACATGCCGCGGTCTCCCGC
GATGGCGGATCAGGCGCGCTTTGAGTTGGCGCTGGGCGAGATGCCCGGCGCATCGGCCCCGAACGGGGCGATCGCCCCGG
CACCGGCCGAAGGCCCGGCGGCGCCGGTCGCGCTCGGCGAGCCGCTGGGCCGCCGCATTCTTGGACAGTTGCGCGGCGGC
CTGGCCGATGTGGCAGGAAAATGGCGGGCGGTGCAGACGGGCTTGGCCGAGGTGAGCCAGGCGCCTACCGTGGTGGGTAT
GCTCGATCTGCAGGCCAGGTTGCTACAGGCATCCGTGGAGTACGAGTTGGTGGGCAAGGCAATAGGGCGCGCCACCCATA
ACGTCTACACGCTGGCGAGAATGTCATGA

FIG. 28

BSCJ

ATGAACGCCATCGGGGCGATCCAACGGTATCGGCGCGGCGCGGGATGGGCGGCCCTGGCGCTCGCCCTGGCGCTGCTGGC
CGGCTGCGGCGCCCGCGTCGAGCTGTTGGGCGCGGCGCCCGAGAACGAAGCCAACGAAGTATTGGCGGCGCTGCTCGAGG
CAGGCATCGCTGCGCAGAAGCAGTCCGGCAAGGCCGGCTACGCGGTTTCGGTGCCGGCCGAGGCGGTGGCCCGGTCGCTG
GAGATCCTGCGCGCAAGCGGCCTGCCCCGCGAGCAGTTCGACGGAATGGGGCGCATATTCCGCAAGGAAGGCCTGGTTTC
ATCGCCGCTCGAAGAGCGCGCCCGCTACATTTATGCGCTATCTCAGGAATTGGCCGACACCCTGTCGCAGATCGACGGCG
TGCTCAGCGCCCGCGTGCACGTGGTGCTTCCCGAGCGCGGCGCGGTCGGCGAGCCGGCCACCCCTTCGACGGCAGGGGTG
TTTCTCAAGTACCGCGACGGACAGAGCCTCGACGCGCTCGTGCCCGAGATCCGCAAGCTGGTCACGCATGCCATCCCGGG
CCTGGCCGAGGACCGTGTATCGGTTGCCCTGGTGGTGGCCCAGCCCGTTCAGGCCGCACCCGTGCCGGTCGCGTGGCGCC
GCGTGCTTGGCGTACAAGTCGCGGACGGATCGGTCCTGAGATTTTCGCTGTTGCTGCTGTTGTTGCCGGTGCTGTGCCTG
ATAGTGGCGGGGGCCACGCTCTACGCCTGGCGCACGCGCTGGTCCCGCGGCGAAAGGCGCGGCGGCGCTGGCGCCGGCGC
CACGGAAGGAGCCGGGCATGACTGA

FIG. 29

BSCK

ATGACTGAGAAGAGCGTGCTGCTTTCCGAGCGGCTCATGATATTCAATCTCCTGCCCAGCCTGACCCTGCATGCCAGTCG
CCACGACGAGACGTTTCCAGCCGATTGGGTGCGCGCGTTGTGCAATGCCGACGCGGCGTTGGCCAACGCATGGCATCGCC
ATTGGTCGCGCTGGATCTTGTGCGAGCTGGGCCTGCTGAACCAGCCGGTCCTGAGCCTCGATCCGCCGCAGTTGAAGGTC
GCGCTATTGTCCACGGACGCCTTGCGCACCTGCGCCGCCCATGCGGGAGCGCTGCTGTGCGCGCCGCGCCTGCGACGCGC
GATAGACGGCGCTGAGGTCCGTACCTTGCATGCCGCGCTCGGGCGCGATGTGATGAATTTCGCCGTGTCTTCCGCGGCGC
GGGCCCTGCATGACGGGATCGCCGCCAGTTCGGACTGGACCCTGGCCGCCACGGTCCAGGCGGCGCAGAAACTGGGCTGG
GCCGTGCTGCGCGACGCCGTGCAGGGCGCCGCCGACGAGATAGCGCTGCGTTGCGCGCTGAAGTTGCCGCGCGACCTTGA
TCCCGCGCCCGTCCTGCCGCCCGAGGCGGCGCTTGCGCTGGTGCTGTCCATGCTCGAAATCCTGGATGCCGAATGGCTTT
CCTCGTTCCCCGCCCAAGCCTGA

FIG. 30

BSCL

ATGGCTTTCCTCGTTCCCCGCCCAAGCCTGATCCAGGCGGTACGGCCCGGCCGTGCGGATCCCGCGACCGACGTCTTGCG
CGCCGAAGACTACGCCGAGCTGCTCAGCGCCGCGCAGATCGTTGCCCAGGCACATCGGCGGGCCGACGAAATCGTGGCCG
AGGCGCGAGAGGAGTTCGAGCGCGAGCGCAGGCGAGGCTATGAGGAGGGGCGCCGCGAAGCGCTTACGGATCAGGCGGAG
AAGATGATAGAAACGGTAAGCCGCACGATCGACTACTTCGCGGGTATCGAGAACGAGATGATCGAACTGGTCATGAGCGC
GGTCCGCAAGAACGTCGACGGTTACGACGACCGCGAGCGCACCGTGATCGCCGTGCGCAACGCATTGGCGGTCGTGCGCA
ATCAGCGCCAGATGACCTTGCGCCTGCACCCCGACGCCAGGCTGACGCCGGGAGCCTGCATACTGGAGAGCGAGATAGG
TATCCGGGCGTGGGCTACCTGGACCTGCTGCCCGACGCCTTGCGGGCGGCCTTCGAACGTACATTCGGCCGGCGCGGATAG
CATGGTCGAGGCCAGCCTCGAGGACCAGCTGTGCGCCTTGCGGGCGGCCTTCGAACGTACATTCGGCCGGCGCGGATAG

FIG. 31

BSCN

ATGCGTCAGTACCACTACATCACGGAGATGATGCGGGTGGCCCTGCAGGATCTGTCCACGTTGCGGATAAAGGGCCGAGT
GGTGCAAGTGGTGGGAACGATCATCAAGGCCGTCGTTCCGATGGTCAAGATCGGCGAAGTGTGCCTGCTGCGCAATCCTG
GCGAGGACTTCGAGATGCACGGCGAAGTGGTGGGCTTTGTTCGCGACGCCGCCTTGCTCACGCCCATCGGCGACATGTAC
GGGATTTCCTCGGCGACCGAGGTGATACCGACCGGACGCACGCATATGGTGCCCGTCGGTCCGGGCTTGCTGGGACGCGT
GCTGGACGGGCTGGGACGTCCGCTGGACGTCGCCGAGTCAGGGCCGCTGCATGCCCACAAGTTCTATCCGGTCTTCGCCG
ATGCGCCGGATCCGCTGACGCGTCGCATCATCCATGCTCCGCTGGAGCTGGGGGTGCGCGTACTGGACGGTTTGCTTACA
TGCGGGGAAGGCCAGCGTCTGGGAATTTTCGCAGCAGCCGGCGGCGGCAAGTCGACCTTGCTGGGCATGCTGGTCAAGGG
CGCCGCGGTCGACGTGACGGTGGTGGCGCTGATCGGCGAGCGTGGGCGGGAAGTTCGCGAGTTCCTTGAGCACGAACTCG
GTCCGGAGGGCAGACGCAAGAGCGTGATCGTTTGCGCGACCAGCGACAAGTCCTCGATGGAGCGTGCCAAGGCGGCATAC
GTCGCAACCGCCATCGCCGAATACTTCCGCGATCAAGGGCAGCGTGTACTTTTTCGGATGGATTCGGTCACCCGCTTTGC
GCGAGCCCAGCGTGAAATCGGCTTGGCGGCAGGCGACCCGCCGACGCGGCGCGGCTATCCGCCGTCGGTGTTCGCCACCC
TGCCAAAACTGATGGAGCGCGCCGGCATGAACCAGACGGGTTCGATCACGGCGCTGTATACGGTGCTGGTCGAGGGGGAC
GACATGAACGAACCGGTGGCCGACGAGACGCGTTCGATACTGGACGGCCACATCGTGCTCTCGCGCAAGCTGGGAGCGGC
GAATCACTATCCTGCCGTCGACGTCCTGGCCTCAGCCAGCCGGGTCATGAATGCCGTGGTGTCGCCACGTCACAAGTACC
TGGCCGGACGTATGCGCGAACTGATGGCCAAGTACCAGGACGTCGAGCTGTTGGTGAAAATCGGCGAGTACAAGCAGGGC
GCCGATGCGTCGACCGATGAGGCGATACAGAAGATCGGACAGATCAATGCGTTTCTCAGACAACTAACCGACGAACGCGA
AGCATTCGAGGATACCGTACTGCGCATGGCTGAAATCATCGGACCCGAATCCTAA

FIG. 32

BSCO

ATGGACCTGGAAAGCCTGCTTGCCATCAAGCATTTTCGCGCCGACCAAGCCCAGCTTGCGCTGAAACGCCAACAGCAGGC
CTGCGCGGTTGCTGCCGCGGCGCAGCGCCAGGCGCAAGGCCGCCTCGACAATTGTCGCCTGTGGGCCGGGCAGCTCGAAA
ACCGTCTTTATGCCGAGCTGTGCCGGCGCATCGTCAAGACACGCGACATCGACGAGGTGCTGCAACGAGTGGGCCACGCC
CGCGACCGCCAGGCCAGCCTGGCGCTGCAGCTCGACAACGCCGTGCGCCGCCACGAGCATGAAATCCAGCTGCTCGCGCA
GCAGCGCGAGCAGCACCGGGAGTGCTTCCAGGCGCAGCAACGGATCGCCGAGTTGGTGCGCCTGCAGCAGGTCGAGGCGG
CGGCCTTGCGCGAGAGCCTGGAGGATCTCTAA

FIG. 33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10690

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 39/10, 39/02, 39/00; A01N 63/00

US CL :424/240.1, 234.1, 253.1, 254.1, 184.1, 93.2, 93.4; 93.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/240.1, 234.1, 253.1, 254.1, 184.1, 93.2, 93.4; 93.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	YUK et al. The BvgAS virulence control system regulates type III secretion in Bordetella bronchiseptica. Mol. Microbiol. June 1998, Vol. 28, No. 5, pages 945-959, see entire document.	1-20 and 47
Y	WOESTYN et al. YScN, the putative energizer of the Yersinia Yop secretion machinery. J. Bacteriol. March 1994, Vol. 176, No. 6, pages 1561-1569, see entire document.	1-10
Y, P	EP 0 889 120 A1 (IMPERIAL COLLEGE INNOVATIONS LIMITED) 07 January 1999, see entire document.	1-20 and 47

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
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"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
24 SEPTEMBER 1999

Date of mailing of the international search report
22 OCT 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
S. DEVI
JOYCE BRIDGERS
PARALEGAL SPECIALIST
CHEMICAL MATTERS
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

BNSDOCID: <WO___9959630A1_IA>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10690

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HENSEL et al. Functional analysis of ssaJ and the ssaK/U operon, 13 genes encoding components of the type III secretion apparatus of Salmonella Pathogenicity Island 2. Mol. Microbiol. 1997, Vol. 24, No. 1, pages 155-167, see entire document.	1-10
Y	BOLAND et al. Role of YopP in suppression of tumor necrosis factor alpha release by macrophages during Yersinia infection. Infect. Immun. May 1998, Vol. 66, No. 5, pages 1878-1884, see entire document.	1-10
A	COTTER et al. BvgAS-mediated signal transduction: Analysis of phase-locked regulatory mutants of Bordetella bronchiseptica in a rabbit model. Infect. Immun. August 1994, Vol. 62, No. 8, pages 3381-3390, see entire abstract.	1-20 and 47
A	COTTER et al. A mutation in the Bordetella bronchiseptica bvgS gene results in reduced virulence and increased resistance to starvation, and identifies a new class of Bvg-regulated antigens. Mol. Microbiol. 1997, Vol. 24, No. 4, pages 671-685, see entire document.	1-20 and 47
A	TEJADA et al. Comparative analysis of the virulence control systems of Bordetella pertussis and Bordetella bronchiseptica. Mol. Microbiol. 1996, Vol. 22, No. 5, pages 895-908, see entire document.	1-20 and 47
A	LEE CA. Type III secretion systems: machines to deliver bacterial proteins into eukaryotic cells? Trend. Microbiol. April 1997, Vol. 5, No. 4, pages 148-156, see entire document.	1-20 and 47
A, P	GALAN et al. Type III secretion machines: Bacterial devices for protein delivery into host cells. Science, 21 May 1999, Vol. 284, pages 1322-1328, see entire document.	1-20 and 47

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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-20 and 47

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG, MEDLINE, EMBASE, BIOSIS, TOXLINE, AGRICOLA, FEDRIP, PASCAL, JAPIO, DERWENT, JICTEPLUS, Inside Conferences and Dissertation Abstracts.
Serach terms: Bordetella, type III secretion mutant, bscN, bsp22, inventors' names

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claim(s) 1-20 and 47(1), drawn to a genetically engineered secretion system *Bordetella* mutant, a vaccine comprising the same and a method for vaccinating using the mutant.
Group II, claim(s) 40-46 and 47(2) (i.e. second claim numbered 47), drawn to a live mucosal antigen delivery vector, a vaccine comprising the same and a method for vaccinating using the same.
Group III, claim(s) 21-39 and 48-51, drawn to a purified nucleic acid comprising a sequence encoding a *Bordetella* type III secretion system component, a polypeptide encoded by a nucleic acid, a DNA molecule encoding a polypeptide and a method for cloning a DNA molecule.
The inventions listed as Groups I, II and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of invention I is a genetically engineered type III secretion system *Bordetella* mutant, a line attenuated vaccine component against *Bordetella* comprising the mutant and a method for vaccinating an animal by administering the mutant. Individually, the product(s) and the first method of use of the product(s) are a permitted combination of categories under PCT Rule 13.2. Invention II is directed to a second product and a method for its use, i.e., a live mucosal antigen-delivery vector, a vaccine comprising the same and a method of immunization. Invention III is directed to a third product, a purified nucleic acid comprising a sequence, a polypeptide encoded by the nucleic acid sequence and a method of cloning a DNA molecule. The three products are structurally, functionally and biologically distinct. The special technical features of the inventions are not linked and therefore the inventions lack unity.